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13. ABSTRACT (Maximum 200 Words)

The goal of this project was the development of adenoviruses (conditionally-replicating adenoviruses, 'CRA') that will more selectively infect, replicate in, and kill prostate tumor cells. There were three Aims (Tasks): 1) evaluate existing Ad serotypes for their possible utility, 2) use phage display technology to isolate prostate tumor-binding peptides, and 3) to combine the best targeting strategy identifiedwith prostate tumor-selective Ad replication. We made substantial progress in Aims 1 and 2, and identified two promising native fiber proteins for further use. Our work with the synthetic prostate tumor-binding peptides was in the end less successful. While a number of peptides that bound the cells were identified, none of these proved to be of utility in the context of the adenoviral fiber protein. Aim 3 was designed to use the fiber(s) identified in the first two Aims in construction of a replicating adenovirus driven by the osteocalcin promoter. We isolated this promoter and constructed plasmids in which the viral E1a gene or beta-galactosidase was driven by osteocalcin, for use in virus cconstruction. In contrast to numerous previous reports, we did not see significant promoter activity following transfection of cells. Replicating viruses with this promoter were not constructed

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Table of Contents

Introduction

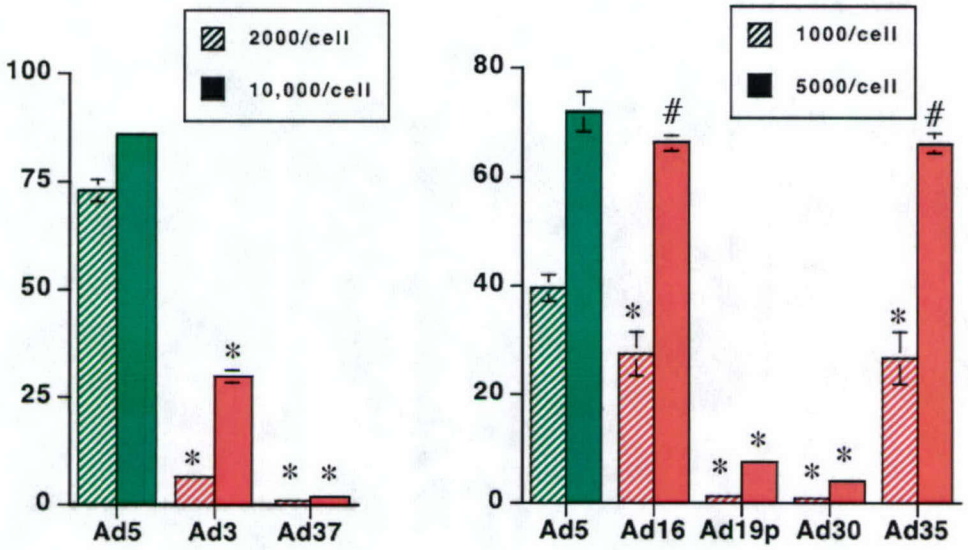
The overall goal of this project was the development of adenoviruses (conditionally-replicating adenoviruses, 'CRA') that will selectively infect, replicate in, and kill prostate tumor cells, with the spread of progeny virus to further tumor cells. While CRA have been described before, all have been based on the tropism of the commonly used adenovirus type 5 (Ad5), which 1) does not efficiently infect many tumor cells, and 2) infects a wide variety of normal cells. Both of these issues are likely to reduce the efficacy of treatment and lead to increased toxicity. By improving infection of the tumor cells and the selectivity of conditionally replicating viruses, we hope to improve the therapeutic window associated with replicating Ad treatment. There are three Aims (Tasks) in the project: 1) to evaluate existing Ad serotypes for their possible utility, 2) to use phage display technology to isolate peptides that selectively bind to prostate tumor cells, and 3) to combine the best targeting strategy produced in the first two aims with prostate tumor-selective Ad replication. In years One and Two of the proposal, work was planned for only the first two Aims. We made substantial progress in both and identified two promising native fiber proteins for further use. Our results with the synthetic prostate tumor-binding peptides was in the end less successful. While a number of peptides that bound the cells were identified, none of these proved to be of utility in the context of the adenoviral fiber protein (see 2003 Annual report.

Based on these results, we chose to proceed with the native fiber approach. We identified the Ad16 and Ad35 fibers as being particularly effective at binding/infecting the prostate tumor lines of interest, and elected to proceed with making recombinant Ads using these proteins. While this part of the work was ongoing, publications from other groups showed that both of these fibers bind the same cellular receptor (CD46) and that it is present on most human cells. We therefore chose to concentrate on the Ad16 fiber rather than to pursue essentially duplicative work with the Ad35 protein as well. Aim 3 was designed to use the fiber(s) identified in the first two Aims in construction of a replicating adenovirus driven by the osteocalcin promoter. We isolated this promoter and constructed plasmids in which the viral E1a gene is driven by osteocalcin, for use in virus cconstruction. In parallel, we assessed its activity in prostate tumor cells using a betagalactosidase reporter gene. Surprisingly, and in contrast to numerous previous reports, we did not see significant promoter activity following transfection of cells. Replicating viruses with this promoter have not been successfully constructed.

Body

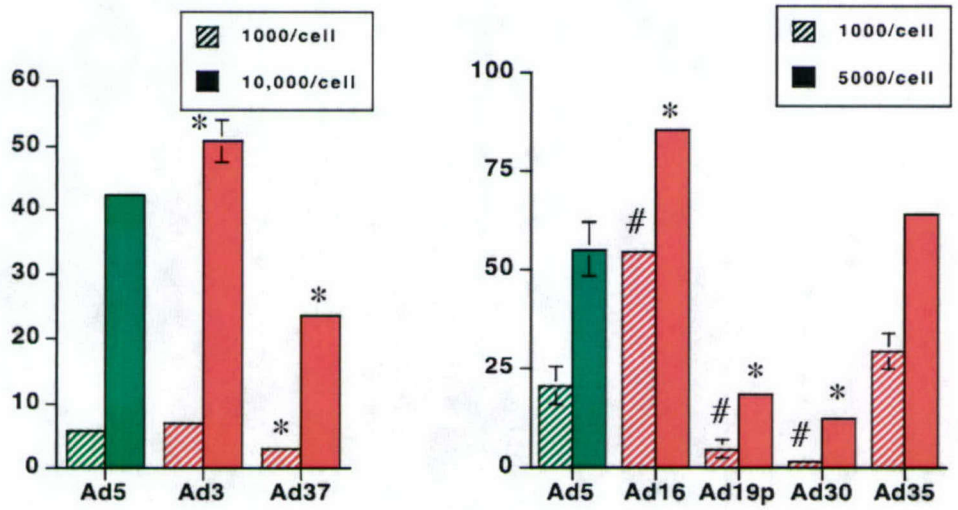
Task 1: Evaluate fiber proteins encoded by the naturally occurring human adenoviruses for their ability to infect prostate tumor cells (months 1-18).

Infection of LNCaP cells



Fiber protein

Infection of DU145 Cells



Fiber protein

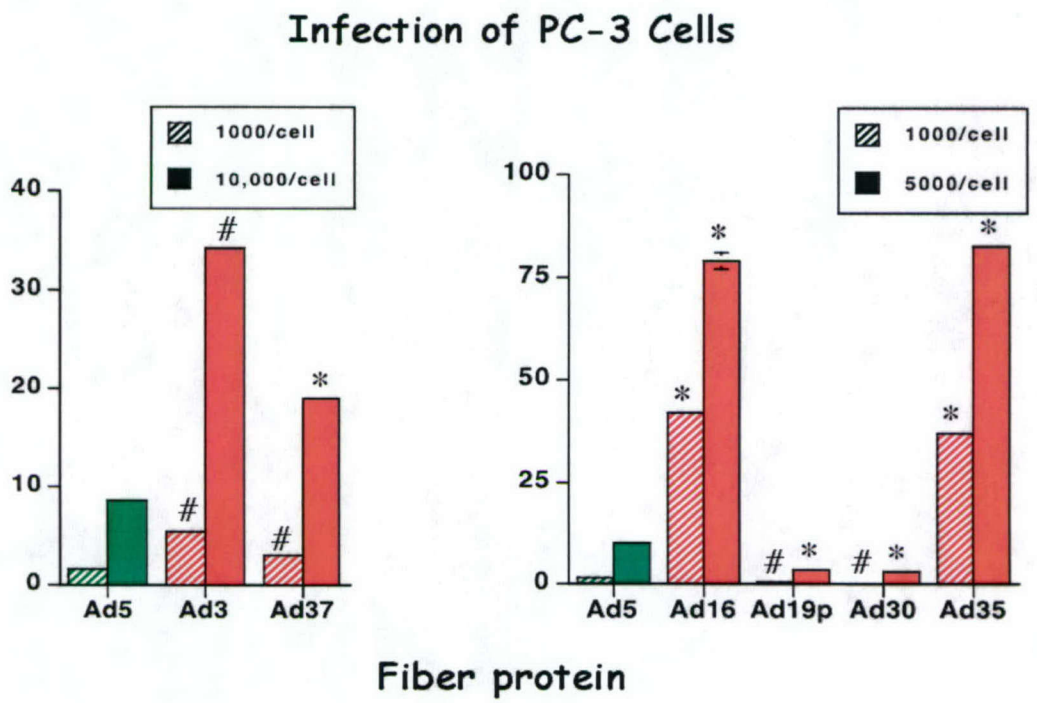


Fig. 1. Infection of prostate tumor lines by the pseudotyped Ads. Cells were infected with virus pseudotyped with the indicated fiber at the particle/cell ratios given. To facilitate interpretation, infection is always compared that produced by Ad5-pseudotyped particles at an equal multiplicity of infection. Statistical significance vs. Ad5: '*', $p < .05$; '#', $p < .10$ (Student's two-tailed T test).

In Year 1, we identified the Ad16 and Ad35 fibers as having the broadest abilities to infect prostate tumor cells (see Fig. 1, above, and 2002 Progress Report). Work by other groups subsequently showed that these fibers bind the same cellular receptor. Work with the Ad35 fiber was therefore abandoned as duplicative, and we chose to concentrate on the Ad16 fiber protein.

Task 2: Use a phage display system to identify peptides that can be used for targeting adenovirus vectors to prostate tumor cells (months 1-24)

As reported in the 2002 and 2003 annual reports, we recovered a number of tumor cell-binding peptides. these peptides were incorporated into the Ad5 fiber and the modified fibers incorporated into Ad particles. The resulting infectivity for prostate tumor cells was assessed as discussed below.

Three motifs (HAIYPRH, SILPYPY, and SAVHLSA) were recovered multiple times in Experiment 1. In *in vitro* assays, all three bound significantly better than unselected library phage to the PC-3 cells, and more poorly than the library phage to the HepG2 cells. We extended the in vitro binding to other prostate tumor cell lines, and found that phage expressing the HAIYPRH and SAVHLSA peptides were able to bind to DU145, LNCaP, and PC-3 cells. In experiment #2 three peptide sequences (NGYSWTS, MGTPPWR, and MILPQKV) were recovered multiple times, accounting for the majority (79%) of sequenced clones. Of these,

NGYSWTS and MILPOKV proved to have the most favorable binding to the tumor cells (see 2002 and 2003 Annual Reports).

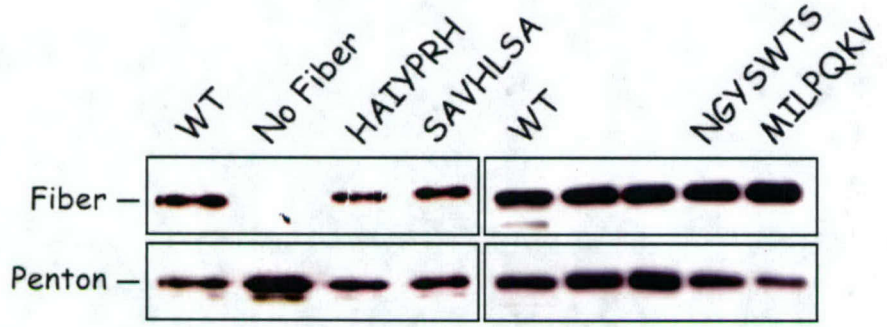


Fig. 2: Generation of Ad particles with peptide-modified fiber proteins. 293T cells were transfected with expression constructs encoding the various fiber proteins, and 24 hours later infected with the fiber-deleted Ad5 vector Ad5.GFPΔF. Approximately 48 hours postinfection, virus was harvested and purified by CsC1 gradient ultracentrifugation. Five micrograms of each viral preparation was then subjected to Western blot analysis. Blots were probed with polyclonal antibodies against the fiber (upper panels) or penton base (loading control; lower panels) proteins. Lanes marked 'wt' contain a first-generation Ad5 vector as a standard. Lanes containing peptide-modified fibers are identified by the peptide sequence inserted into the fiber protein.

Addition of the recovered peptides to a 'detargeted fiber protein

The peptides were inserted into a genetically 'detargeted ' Ad5 fiber protein which lacks CAR binding due to the presence of a double point mutation (KO1; Jakubczak et al. 2001) J. Virology **75**:2972-2981). We have inserted a short linker sequence and a unique restriction site for insertion of the peptide sequences into the HI loop of this fiber (Nicklin et al. (2001) Molecular Therapy **4**(6): 534-542)

Ad particles with the modified proteins were then generated using a rapid transfection/infection protocol. We found that all of the resulting proteins formed stable trimers and assembled onto particles at essentially normal levels (Fig. 2). These viral preparations were then tested for their ability to infect prostate tumor cells as well as non-prostate derived cells (Figs. 3 and 4).

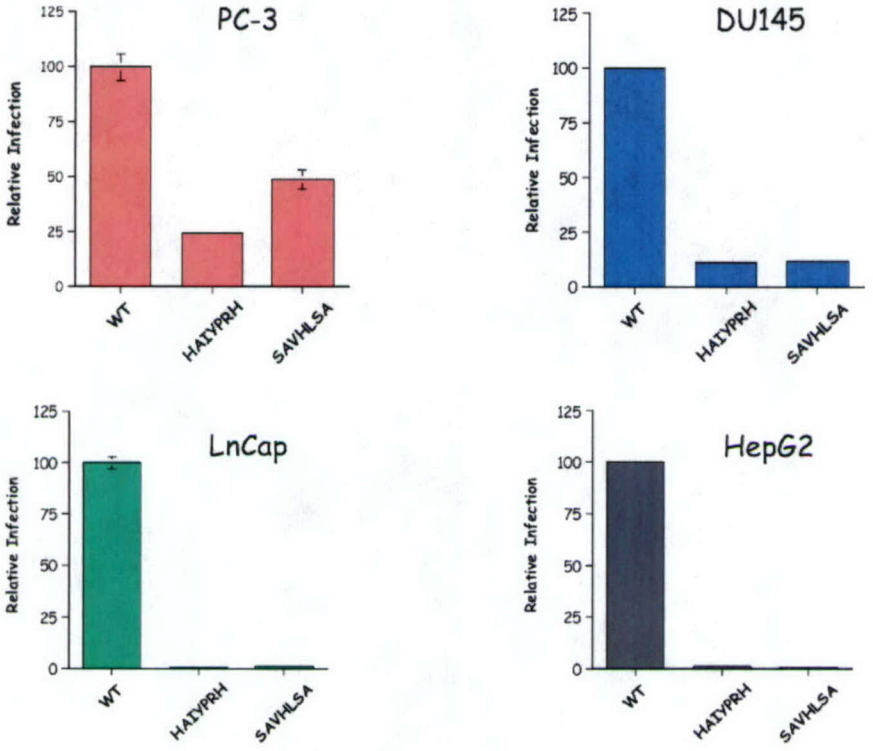


Fig. 3. Infection of prostate tumor cell lines by Ads targeted with peptides recovered in screen #1. Cells were incubated for three hours with 5000 particles/cell of Ad5.GFP.ΔF equipped with the wt) fibers, or with the KO1 fiber with HI loop insertion of HAIPRYH or SAVHLSA peptides. Twenty four hours post-infection, GFP fluorescence was assessed by FACS. Fraction of GFP-positive cells are expressed relative to the percent GFP-positive using the Ad5 fiber.

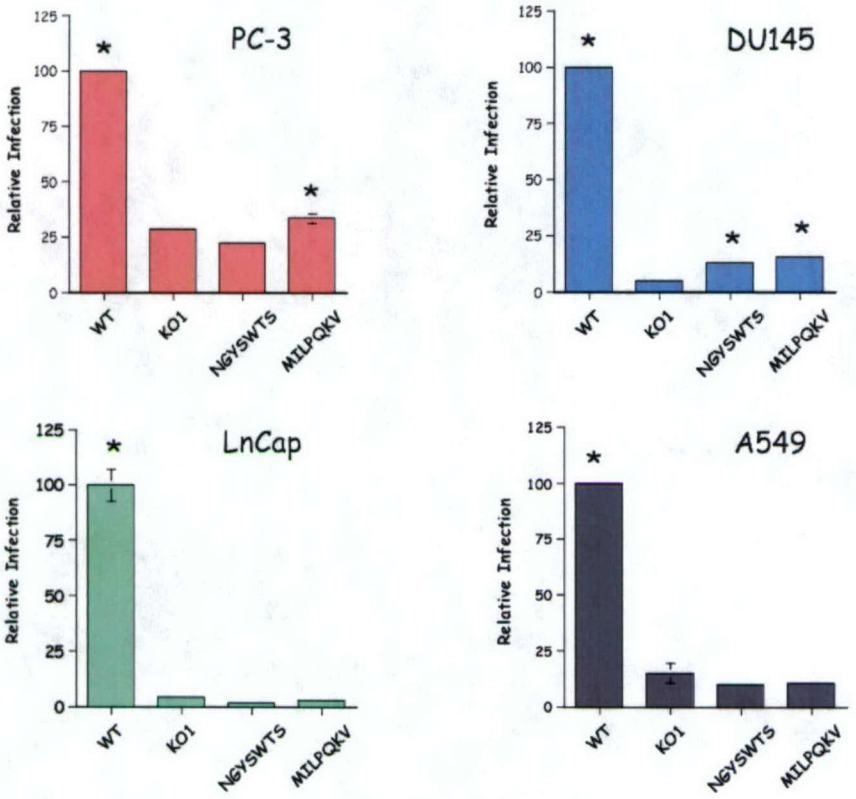


Fig. 4. Infection of prostate tumor cell lines by Ads targeted with peptides recovered in screen #2. Cells were incubated for three hours with 5000 particles/cell of Ad5.GFP.ΔF equipped with the wt or K01 (detargeted) fibers, or with the K01 fiber with HI loop insertion of NGYSWTS or MILPOKV peptides. Twenty four hours post-infection, GFP fluorescence was assessed by FACS. Fraction of GFP-positive cells are expressed relative to the percent GFP-positive using the Ad5 fiber. '*': $p<0.05$ from K01 (Student's T test)

In summary, the results from panning of the phage library have been relatively disappointing. The two best peptides evaluated from Experiment #2 (NGYSWTS and MILPQKV) did confer significantly improved infection of PC-3 and DU-145 cells relative to the detargeted fiber lacking a peptide insert (Fig. 4), but it is unclear if this level of infection will be sufficient for eventual use in a targeted vector. Additionally, SAVHLSA was able to confer increased infection on PC-3 but not other cell lines (Fig. 3), suggesting that it does not bind a receptor generally expressed on tumor cells.

Based on these results, we chose to concentrate on the native fiber approach to improving infection of prostate tumor cells, using the Ad16 fiber (see Task 1).

Task 3: Construct and test a conditionally-replicating adenovirus incorporating the most prostate tumor-specific fiber protein identified in (1 and 2) for use in anti-tumor therapy (months 25-36)

As noted above, we identified the Ad16 and Ad35 fibers as the most promising natural fibers and are in the process of constructing and evaluating reporter gene-marked Ads with these fibers in substituted for that of Ad5. Because of the use of CD46 by both of these fibers, we have dropped further work with the Ad35 protein and concentrated on the Ad16 fiber. A plasmid with the Ad16 fiber substituted for that of Ad5 was constructed and used for generation of recombinant adenoviruses. This should be suitable for generation of replicating adenoviruses with the tropism of Ad16, which are expected to show much better infectivity for prostate tumor cells than unmodified Ad5 vectors. A virus with the Ad16 fiber and containing the EGFP reporter gene was successfully constructed and has the expected infectivity (see 2003 Report and Fig. 5). This virus was instrumental in another aspect of our work, namely infection of dendritic cells and endothelial cells, and was used in work leading to two submitted manuscripts.

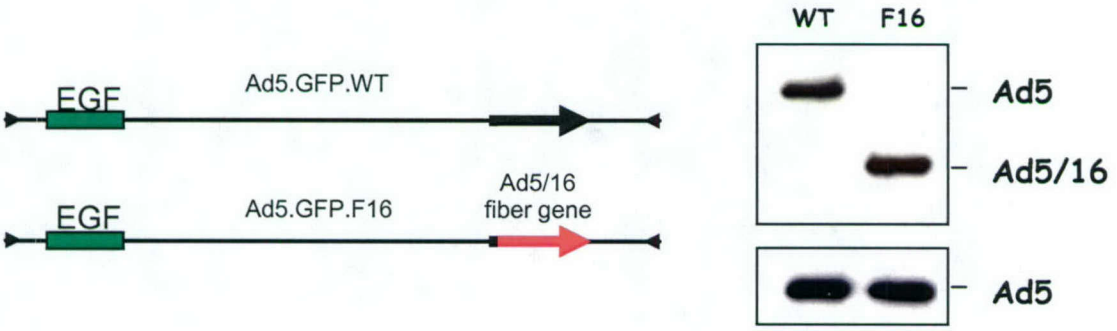


Fig. 5: A) Viruses with the chimeric Ad5/16 fiber gene in place of the wt Ad5 gene. Both Ads express EGFP from the CMV immediate/early promoter. **B**) Western blot of viral particles. Ad5.GFP.WT ('WT') and Ad5.GFP.F16 ('F16') were propagated in 293 cells and CsCl-purified particles were analyzed for fiber content using a monoclonal antibody recognizing an epitope in the fiber tail. As a loading control, the blot was reprobed with an antibody against the Ad5 penton base.

The other part of this Aim was less successful. Based on much previous literature, we selected the osteocalcin promoter as a candidate for directing tumor-specific expression of the viral E1a protein. We constructed plasmids with the viral E1a region incorporated downstream of this promoter or of the constitutive CMV promoter, which are available for generation of adenoviruses. In parallel with this work, we placed the beta-galactosidase reporter gene downstream of both promoters and assessed betagal activity after transfection of prostate tumor and non-prostate tumor cells. Unexpectedly, we have so far not detected activity of the osteocalcin promoter in any cell line tested. This is in contrast to the published work, and we do not understand the reason for the apparent inactivity of the promoter. Due to time considerations (the funding period has expired and I am closing the lab due to leaving TSRI) this has not been pursued further.

Key Research Accomplishments

- Identification of the Ad16 and Ad35 fibers as prostate cancer-tropic.

- Construction of plasmids for generation of Ad16-targeted virus, and validation of their utility.

- Addition of tumor cell-binding peptides to the HI loop of the Ad5 fiber.

- Generation and evaluation of viruses containing the peptide-modified fiber proteins.

Reportable Outcomes

The viruses with the Ad16 or Ad35 fibers substituted into the chromosomes are valuable reagents for studies of the tropism of these Ad16 in general as well as in the work covered under this proposal.

This work contributed to the successful demonstration that Ad16-targeted virus can be used to infect dendritic cells in vitro, and to the evaluation of its in vivo activity as a vaccine agent (see attached manuscript by Hsu et al., and final report for DAMD17-01-1-0391).

The pseudotyped viruses generated were also used as part of a collaborative study with another group to examine endothelial cell infection by retargeted Ads (see attached manuscript by Denby et al.).

Conclusions

Progress has been made on evaluation of both targeting strategies. We have identified two native fiber proteins (Ad16 and Ad35) that infect a panel of prostate tumor cell lines much more effectively than do the standard Ad5 vectors, and constructed viruses with these fibers substituted for that of Ad5 in the viral chromosome. Our phage display work did not yield peptide targeting that was better than what can be achieved using native fibers. For reasons that we do not completely understand, we found that the osteocalcin promoter was not active in the prostate tumor cell lines tested. Replicating viruses with this promoter were not constructed.

Publications

Hsu, C., Boysen, M.M., Gritton, L.D., Frosst, P.D., Nemerow, G.R., and D. J. Von Seggern. *In vitro* Dendritic Cell Infection by Pseudotyped Adenoviral Vectors Does not Correlate with their *in vivo* Imunogenicity. Manuscript submitted.

Denby, L., Nicklin, S.A., Graham, D., **Von Seggern, D.**J., and A.H. Baker. In vitro and in vivo characterization of Novel Adenoviral Vectors From Subgroup D (Ad19p and Ad37) with a Restricted Hepatic Tropism. Manuscript submitted.

Wu, E., Trauger, S.A., Pache, L., Mullen, T.-M., **Von Seggern, D.J**., Suizdak, G., and G.R. Nemerow. (2004). Membrane Cofactor Protein (MCP; CD46) is a Receptor for Adenoviruses Associated with Epidemic Keratoconjuncitivitis. Journal of Virology, in press

Von Seggern, D. J., Aguilar, H. E., Kinder, K., Fleck, S. K., Gonzalez Armas, J.C., Stevenson, S. C., Ghazal, P., Nemerow, G. R., and M. Friedlander. (2003). *In vivo* Transduction of Photoreceptors or Ciliary Body by Intravitreal Injection of Pseudotyped Adenovirus Vectors. Molecular Therapy **7**:27-34.

Conference Presentations

Hsu, C., Aguilar, E., Friedlander, M., Nemerow, G.R., Nicklin, S.A., White, S., Work, L.M., Baker, A.H., and **D.J. Von Seggern**. (2003). "5 Years of Pseudotyped Adenoviridae: What Have We Learned About Retargeting?: 50 Years of Adenoviridae: Structure, Biology, Vectorology. Montpellier, France.

Hsu, C., Gritton, L.G., and **D.J. Von Seggern.** (2002). Differential Transduction of Prostate Tumor Cells by Adenovirus Type 5 Vectors Pseudotyped with Fiber Proteins from Subgroups B, C, and D Viruses. American Society of Gene Therapy Annual Meeting, Boston, MA.

Appendicies:

1) Meeting Abstracts: 2003 "50 years of Adenoviridae: structure, biology & vectorology
Montpelier, Rrance (Hsu et al.)
2002 ASGT Meeting (Hsu et al.)

2. Publications:

A. Hsu, C., Boysen, M.M., Gritton, L.D., Frosst, P.D., Nemerow, G.R., and D. J. Von Seggern. *In vitro* Dendritic Cell Infection by Pseudotyped Adenoviral Vectors Does not Correlate with their *in vivo* Imunogenicity. Manuscript submitted.

B. Denby, L., Nicklin, S.A., Graham, D., **Von Seggern, D.**J., and A.H. Baker. In vitro and in vivo characterization of Novel Adenoviral Vectors From Subgroup D (Ad19p and Ad37) with a Restricted Hepatic Tropism. Manuscript submitted.

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Adenoviruses Associated with Epidemic Keratoconjuncitivitis. Journal of Virology, in press

D **Von Seggern, D. J.**, Aguilar, H. E., Kinder, K., Fleck, S. K., Gonzalez Armas, J.C., Stevenson, S. C., Ghazal, P., Nemerow, G. R., and M. Friedlander. (2003). *In vivo* Transduction of Photoreceptors or Ciliary Body by Intravitreal Injection of Pseudotyped Adenovirus Vectors. Molecular Therapy **7**:27-34.

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Dan Von Seggern 50% 6/1/2001 - 5/31/2004

Catherine Hsu 50% 10/2001 - 5/31/2004

Megan Boysen 50% 6/2002 - 8/30/2002

Differential Transduction of Prostate Tumor Cells by Adenovirus Type 5 Vectors Pseudotyped with Fiber Proteins from Subgroup B, C, and D Viruses.

Catherine Hsu, Lance D. Gritton, and Dan J. Von Seggern. Department of Immunology, The Scripps Research Institute, La Jolla, CA.

Many gene therapy strategies for prostate cancer are under development, including delivery of tumor suppressor or suicide genes and the use of oncolytic (replication-competent) adenoviruses. The use of conditionally replicating viruses in which viral replication is controlled by a prostate-specific promoter shows particular promise. In all of these strategies, it will be critical to ensure that the virus used infect tumor cells as efficiently and selectively as possible. More than 50 Ad serotypes are known to infect humans, but only one (Ad5) has been widely investigated as a gene transfer vector. Adenoviral tropism is principally determined by binding of the viral fiber protein to its receptor on the target cell, and the receptor for Ad5 is a widely expressed Ig-domain containing protein termed CAR (Coxsackievirus Adenovirus Receptor). Receptor usage by most other Ads remains unknown, but evidence suggests that a fairly large number of non-CAR receptors may exist. CAR's broad expression allows Ad5 to be used in many different situations, but also limits the selectivity of gene delivery. In particular, Ad5 very efficiently infects the hepatocytes, which is undesirable from a therapeutic viewpoint. Recent work has also shown that CAR is not necessarily expressed on all cells within a tumor, and it has been suggested that some of the more aggressive cells may lack CAR expression. We previously developed an Ad pseudotyping system that allows production of vector particles with different or modified fiber proteins. Using the Ad3 or Ad37 fibers, gene delivery could be extended to cells refractory to Ad5 transduction such as EBV-infected B cells and retinal photoreceptors. Using this technique, we are now evaluating the ability of fibers from different Ad serotypes to direct transduction of PC cells. Viral particles containing fibers from Ads of Subgroups B (Ad3, Ad16), C (Ad5), and D (Ad19p, Ad37) were tested on a panel of prostate tumor cells including PC-3, DU145, and LnCaP. In general, the cell lines varied in their susceptibility to infection by any given serotype (including Ad5), indicating heterogeneity of receptor expression. Vector particles equipped with the Ad3, Ad37 or Ad19p fibers were generally less effective than Ad5, while the Ad16 fiber conferred a generally higher level of tumor cell transduction. Results of these studies, as well as studies with further Group B and D serotypes, will be presented.

50 years of Adenoviridae: structure, biology & vectorology
10-12th September, 2003
Saint-Martin de Londres - Montpellier - France
ABSTRACT SUBMISSION FORM
Deadline for submission of abstracts: **25th August, 2003.**

5 years of pseudotyped Adenoviridae: What have we learned about retargeting?

C. Hsu[1], E. Aguilar[2], M. Friedlander[2], G.R. Nemerow[1], S.A. Nicklin[3], S.J. White[4], L.M. Work[3], A.H. Baker[3], and D.J. Von Seggern[1].

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Effective retargeting methods would greatly improve prospects for adenoviral gene therapy. Ad tropism is largely determined by the fiber knob, which binds to cell-surface receptor proteins. The Ad5 fiber also binds heparin sulfate glycoproteins via a motif in the shaft, which confers tropism for hepatocytes. We have concentrated on altering viral infectivity by using different or modified fiber proteins. In order to simplify generation and study of retargeted Ads, we developed a fiber-deleted vector system along with fiber-complementing packaging cell lines.

We have examined the use of alternate fiber serotypes in gene delivery to the eye. In many retinal degenerative diseases the affected cell is the retinal photoreceptor (PR), which transduces light into neuronal signals. Standard Ad5 vectors do not infect PR, and we compared infection by Ad5 to that by vectors pseudotyped with the Ad3 and Ad37 fiber proteins (which are known to use receptors other than CAR). After intraocular administration, the Ad37-pseudotyped virions infected mainly photoreceptors along with a few other ocular cell types, in strong contrast to the pattern of Ad5 infection. As well as providing information about Ad37 tropism, this should allow use of Ad in PR-directed gene therapy. This serotype may also be useful in developing vectors for systemic use, as further experiments showed that Ad37-pseudotyped viruses do not infect the liver.

In a different approach, genetic methods were used to both de- and re-target Ad5 vectors. Point mutations in the CAR-binding site were combined with insertion of phage-selected peptide ligands into the HI surface loop of the fiber knob to produce cell type-selective fibers. This general method should be

applicable to essentially any cell type for which a peptide ligand can be identified. Viruses with fibers that target cardiovascular cells have been developed and their *in vivo* performance is being evaluated.

Please consider my abstract for: ☐ ORAL PRESENTATION ☐ POSTER

In vitro Dendritic Cell Infection by Pseudotyped Adenoviral Vectors Does not Correlate With Their *in vivo* Immunogenicity

*Catherine Hsu, Megan Boysen[1], Lance D. Gritton, Phyllis D. Frosst[2], Glen R. Nemerow, and Dan J. Von Seggern**

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Abstract

Expression of antigens in dendritic cells (DC) can stimulate protective immunity against both viral infection and tumor growth, making them important targets for gene therapy. *In vitro*-generated DC are commonly used in gene delivery studies with the assumption that the results will correlate with *in vivo* activity. Adenovirus Type 5 (Ad5) vectors have been widely used with DC, but these cells lack the primary receptor (CAR) used by Ad5 and are poorly infected. We investigated the use of Ad5 vector particles pseudotyped with fibers from other Ad serotypes in DC targeting. Several fiber proteins, including those from Ad16 (Subgroup B) and Ad37 (Subgroup D), conferred dramatically increased *in vitro* infection. Surprisingly, neither dendritic cell infection nor the immune response to an Ad-delivered antigen was improved when the modified viruses were tested *in vivo*. These results underscore the importance of using appropriate animal models in gene delivery studies.

Keywords

Adenovirus; targeting; dendritic cell; in vivo; T cell; vaccine

Introduction

Dendritic cells (DC), the most potent known natural antigen-presenting cells (APCs), have emerged as an important target for gene delivery. Expression of antigens by DC can stimulate protective immunity against both viral infection and tumor growth (Ludewig et al., 1998; Wan et al., 1997). Efficacy has been correlated with the level of antigen expressed by the DC (Okada et al., 2003), which in turn depends on the efficiency of gene transfer. DC generated *in vitro* from bone marrow or from peripheral blood cells are commonly used in targeting studies, based on the assumption that the results will correlate with *in vivo* infection. Vectors based on Adenovirus type 5 (Ad5) have been used for many of these studies (Wan et al., 1997; Tillman et al., 1999) although DC do not express the Ad5 receptor (Coxsackievirus Adenovirus Receptor; CAR) and high multiplicities of infection (m.o.i.) are often needed. Ad vectors that could more efficiently bind and infect DC might therefore be superior as vaccines. Ads bind to cells via interaction of the viral fiber protein's C-terminal 'knob' domain with a cellular receptor, with internalization occurring through a distinct interaction of the viral penton base protein with cellular γ_v integrins (reviewed in Nemerow, 2002). Since viruses from different subgroups bind to distinct fiber receptors, viral tropism can be modified by substitution of fiber proteins (Von Seggern et al., 2000; Rea et al., 2001). We evaluated the use of fibers from a number of Ad serotypes in DC infection. The Ad16 (Subgroup B) and Ad37 (Subgroup D) fibers significantly improved *in vitro* tropism for bone marrow-derived murine DC. Surprisingly, use of these fibers did not increase *in vivo* cellular immune responses against a model antigen (EGFP) expressed by the retargeted viruses or infection of splenic DC over that seen with the unmodified Ad5 vector. Our results in particular suggest that experiments with *in vitro*-generated DC may not be predictive of *in vivo* activity, and in general underscore the importance of validating targeting studies in relevant animal models.

Results

DC infection using pseudotyped Ad vectors. We first surveyed infection of murine bone marrow-derived DC by Ad5 vectors pseudotyped with the fibers from Ad3 (subgroup B1), Ad16 and Ad35 (Subgroup B2), and Ad19p, Ad30, and Ad37 (Subgroup D), based on previous data showing that they used receptors distinct from CAR (Nemerow, 2002; Law et al., 2002; Segerman et al., 2000). Fiber proteins were stably expressed in packaging cells as previously

described (Von Seggern et al., 2000) and the cell lines were used to produce particles of the fiber-deleted Ad5 vector Ad5.GFP.γ F (Fig. 1A and Von Seggern et al., 2000; Wu et al., 2001). Murine bone marrow-derived DC were then infected with the pseudotyped particles. Most of the non CAR-using fibers conferred greater infectivity than the wt Ad5 protein (Fig. 1B). Based on these results, the Ad16 and Ad37 fibers were chosen as representative members of Subgroups B and D for further work.

Construction of Ad vectors with Ad16 or Ad37 fiber genes: Because fiber-deleted viral particles have abnormally high particle/pfu ratios (Von Seggern et al., 2000), and are not suitable for *in vivo* use (our unpublished data), Ad5 vectors with the Ad16 or Ad37 fiber shaft and knob domains substituted into the viral backbone (Ad5.GFP.F16 and Ad5.GFP.F37, respectively; Fig. 2A) were constructed. Ad5.GFP.F37 is maintained in the 633 cell line (Von Seggern et al., 2000) which expresses the Ad5 fiber protein. The resulting particles contain both fiber proteins and can efficiently enter host cells using the Ad5 fiber (Fig. 2B). Virus containing only the virally encoded Ad37 fiber is then generated by growth in 293 cells, which do not express a fiber protein.

The increased DC infectivity conferred by the Ad16 and Ad37 fibers (Fig. 1) was confirmed for Ad5.GFP.F37 and Ad5.GFP.F16 (Fig. 2C and D). In experiments using several independent preparations of each virus, Ad5.GFP.F37 infected from 3 to 68-fold as many DC as did Ad5.GFP.WT ($n = 7$; mean fold increase = 30.8), and Ad5.GFP.F16 infected 4- to 7-fold more cells ($n = 4$; mean fold increase = 5.6). Viability of the cells was not significantly reduced by viral infection under these conditions (Fig. 2E). The increased DC infection by the modified viruses is presumably due to use of fiber receptors other than CAR. Ad37 has been reported to bind sialic acid residues on cell surface proteins (Arnberg et al., 2002), and we investigated whether sialic acid might play a role in infection of murine DC by Ad5.GFP.F37. Cells were treated as previously described (Arnberg et al., 2002) with *Vibrio cholerae* neuraminidase to remove sialic acid residues, and then infected with Ad5.GFP.WT or Ad5.GFP.F37 (Fig. 2F). Infection by the Ad37-pseudotyped vector was not affected, suggesting that sialic acid is not the receptor used on these cells. Neuraminidase treatment actually produced a small but significant ($p < .01$) increase in infection by Ad5.GFP.WT, consistent with the data of Arnberg et al. (2002) who used wildtype Ad5 in a similar experiment.

Immunogenicity of retargeted Ads. To assess the cellular immune responses generated by the retargeted Ads, escalating doses were injected and the responses evaluated using an assay for intracellular IFN-γ production by $CD8^+$ T cells (Fig. 3A). Female BALB/c mice (8/group) were immunized subcutaneously with a single administration of 1×10^8, 1×10^9, or 1×10^{10} particles of each Ad. Four weeks later. splenocytes were harvested and cultured either with the HYLSTQSAL peptide, which has been identified as a major T cell epitope in this strain (Gambotto et al., 2000), or with an irrelevant control peptide (SIINFEKL) derived from ovalbumin (OVA). As shown in Fig. 3B, all viruses were able to generate EGFP-specific T cell responses. At the lowest dose tested (1×10^8 particles/mouse), modest EGFP-specific T cell responses were detected only after administration of the unmodified Ad5 vector. This dose of Ad5.GFP.F16 or Ad5.GFP.F37 did not result in a greater response than that detected against the irrelevant control peptide (Fig. 3C). At higher doses (1×10^9 or 1×10^{10} particles/mouse), the fraction of $CD8^+$ cells reactive for the EGFP peptide was essentially the same for all three vectors. T cells from vehicle-injected animals (Fig. 3A) or naive mice (data not shown) did not respond to stimulation with either peptide.

Serum antibodies against the EGFP transgene product or the Ad capsid itself were also measured by ELISA against either recombinant EGFP or purified viral particles. At all doses tested (1×10^8 - 1×10^{10} particles/animal), antibody levels against either antigen following immunization with Ad5.GFP.F16 or Ad5.GFP.F37 were reduced relative to that seen in animals given Ad5.GFP.WT (data not shown).

***In vivo* DC infection.** *In vivo* infection of splenic DC was assayed essentially as described (Zhang et al., 2001). 1×10^{11} viral particles/mouse was injected via the tail vein, $CD11c^+$ DC were isolated from spleens 24 hours later by magnetic cell sorting, and the fraction that were also positive for EGFP expression was determined by FACS. We found that a small percentage of splenic DC were infected, and that infection was not improved by fiber substitution (Fig. 4A). Infection of DC by Ad5.GFP.F37 did not differ significantly from Ad5.GFP.WT, while significantly fewer ($p = .006$ vs. Ad5.GFP.WT) DC were infected by Ad5.GFP.F16. This experiment was performed twice with similar results.

Discussion

A variety of methods have been developed to improve infection of dendritic cells by adenovirus. Bispecific molecules have been designed to bind both the Ad fiber and proteins expressed on the surface of DC (Pereboev et al., 2004). Vectors pseudotyped with non CAR-using fibers have been shown to have enhanced *in vitro* infectivity towards both human (Rea et al., 2001) and murine (this study) DC. In a genetic approach, CD40 ligand was fused to the fiber protein, with dramatically increased *in vitro* DC infection (Belousova et al., 2003)). However, the *in vivo* utility of the retargeted virus has been evaluated in only a few cases. Incorporation of the integrin-binding RGD motif into the Ad5 fiber knob increased both *in vitro* DC infection and the *in vivo* cellular and humoral immune responses, and improved the generation of anti-tumor immunity (Worgall et al., 2004). In another study, complexing the virus with a bispecific fiber Ab/CD40 ligand protein improved not only *in vitro* DC infection, but also increased *in vivo* generation of antigen-specific T cell responses (Pereboev et al., 2004).

We found that substituting the Ad16 or Ad37 fibers onto the Ad5 capsid resulted in large improvements in both the fraction of DC infected *in vitro* and in the amount of transgene product (EGFP) expressed by the infected cells (Fig. 2). As the three viruses used here differ only in their fiber proteins, differential fiber receptor usage most likely accounts for most of these differences. The identify of the fiber receptor(s) used by these viruses on the murine DC is unclear. Both Ad16 and Ad37 have been reported to use CD46 as a fiber receptor, and Ad37 has also been reported to bind sialic acid (Wu et al., 2004; Gaggar et al., 2003; Arnberg et al., 2002). We were not able to assess expression of CD46 on our DC, due to the lack of suitable antibodies against the murine protein. Experiments in which we used neuraminidase treatment suggest that sialic acid is not the Ad37 receptor being used on DC *in vitro*.

While the increased *in vitro* infection we observed compares favorably with that seen by other groups (Worgall et al., 2004; Pereboev et al., 2004; Rea et al., 2001), it did not translate into increased generation of transgene-specific T cells (Fig. 3) or increased splenic DC infection in vivo (Fig. 4). If DC *in vivo* expressed neither CAR or the receptor(s) for Ad16 or Ad37, then the infection by all three serotypes tested would be largely mediated by integrins and not by fiber. In this case all three of our Ads would have similar *in vivo* infectivities and activities, which is exactly what we observed. This might also explain why the RGD-modified virus used by Worgall et al. (which uses γ_v integrins more effectively than unmodifed Ad5) was able to improve *in vivo* performance (Worgall et al., 2004). Taken together, we think that the simplest

interpretation of our data is that there are important differences between *in vitro*-generated DC and those found *in vivo*. As the cells used here were generated using the same method used in most other reports (Inaba et al., 1998), this study suggests that *in vitro* targeting results should be interpreted with some degree of caution in the absence of confirming *in vivo* data. This is reminiscent of earlier work where *in vitro* studies aimed at understanding liver transduction by Ad5 gave misleading results. CAR and γ_v integrins were identified as the fiber receptor and co-receptor for Ad5 (for review see Nemerow, 2002), and modifying the vectors to ablate use of these receptors provided effective *in vitro* detargeting of cells including hepatocytes. Unexpectedly, viruses with both the CAR- and integrin-binding motifs mutated were still able to transduce the liver *in vivo*. This led to the unanticipated discovery that the fiber shaft is a very important determinant of hepatocyte infection *in vivo*, probably through binding of heparin sulfate glycoproteins (Smith et al., 2003). *In vitro* vaccine targeting studies with promising results should therefore be confirmed using relevant animal models as early as possible.

Methods

Adenoviral pseudotyping: Ad5.GFP.γ F and packaging lines expressing the Ad5, chimeric Ad5/3, and Ad37 fibers have been previously described (Von Seggern et al., 2000; Wu et al., 2001). The shaft/knob portion of the Ad16 fiber gene was amplified from viral DNA using the primers 5' CCG GTC TAC CCA TAT GAA GAT G 3' and 5' TGG TGC GGC CGC TCA GTC ATC TTC TCT G 3'. This fragment was substituted for the corresponding fragment of pDV67 (Von Seggern et al., 2000), creating pDV156, which expresses a fusion protein consisting of the N-terminal 16 aa of the Ad5 protein fused to the C-terminal 338 aa of the Ad16 fiber. The construct for expression of the Ad35 fiber (pDV166) was made using the same strategy and the new 3' oligo 5' TGG TGC GGC CGC TTA GTT GTC GTC TTC TGT AAT G 3'. Plasmids for expression of the Ad19p or Ad30 fibers (pDV145 and pDV164, respectively) were constructed exactly as described for pDV121 (Wu et al., 2001) except that the fiber genes were amplified from Ad19p or Ad30 genomic DNA. Cell lines for fiber expression were generated as described (Von Seggern et al., 2000). Ad5.GFP.γ F was grown in cell lines expressing the fiber of interest and the particles purified by CsCl gradient centrifugation and analyzed by western blotting as described (Von Seggern et al., 2000).

Virus construction: To construct Ad5.GFP.F16 and Ad5.GFP.F37, derivatives of pAdEasy (QBiogene) with the Ad16 or Ad37 fiber shaft and knob domains in place of the Ad5 sequences were constructed (pDV182 and pDV158, respectively). Briefly, the portion of the fiber genes from the site corresponding to the Sph1 site (nt 180 of Ad5 fiber) to the stop codon were amplified and substituted for that of Ad5 (full construction details are available on request). The fusion proteins therefore consist of the first 59 aa of the Ad5 protein fused to the shaft and knob domains of the Ad16 or Ad37 fibers. Plasmids containing full-length Ad5.GFP.F16 (pDV189) or Ad5.GFP.F37 (pHY1) genomes were generated by allowing pDV182 or pDV158 to recombine with pAdTrack in E. coli strain BJ5183 (QBiogene), and the resulting plasmids transfected to 633 cells. Virus for experiments was produced in 293 cells and purified by CsCl gradient (all virus for use in animals was purified by two sequential CsCl gradients), dialyzed into 40 mM TRIS-8.1, 0.9% NaCl, and 10% glycerol, and stored at -70 °C.

Dendritic Cells: DC were generated by culture of bone marrow from 8 week old female BALB/C mice in the presence of 10 ng/ml each murine GM-CSF and IL-4 (R & D Systems, Minneapolis, MN) (Inaba et al., 1998). Cells were stained with antibodies against MHC Class II, CD3, CD80, CD86 (eBiosciences, San Diego, CA) and CD11c (Pharmingen, San Diego, CA) and assayed by FACS, and were routinely < 2 % CD3+, >80% CD11c+, >20% CD80+, >15 % CD86+, and > 80 % MHCII +. For infection assays, cells were incubated with virus in RPMI + GM-CSF and IL-4, and the percentage of GFP-positive cells determined 24 hours later by FACS. For removal of sialic acid, DC were incubated with 10 mU/ml of *Vibrio cholerae* Type III neuraminidase (Sigma) for one hour in PBS with 2% fetal calf serum. Cells were then washed with complete RPMI and infected as described above.

IFN-γ release assay: Spleens were isolated from mice and mechanically disrupted. Following lysis of red blood cells, 1 x 10^6 splenocytes were cultured for three hours in RPMI with Golgiplug (BD Biosciences) in the presence or absence of 0.1 μg/ml EGFP epitope peptide HYLSTQSAL (Gambotto et al., 2000) or the irrelevant ovalalbumin-derived peptide SIINFEKL as a control. Peptides were synthesized by the TSRI Protein and Nucleic Acids Core Facility. Cells were stained with an anti-CD8 antibody (eBioscience), fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and stained with an antibody against IFN-γ (eBiosciences, San Diego, CA). The cultures were then analyzed by FACS and the percentage

of the CD8+ cells positive for IFN-γ was determined ((number of CD8+ IFN-γ+ cells/ total CD8+cells) x 100).

***In vivo* dendritic cell infection**: Mice (4/group) were injected via the tail vein with 1 x 10^{11} particles of the indicated virus in a volume of 100 μl. 24 hours later, spleens were harvested, single-cell suspensions were generated by collagenase/dispase treatment, and CD11c+ cells isolated using anti-CD11c magnetic beads (Miltenyi). The recovered cells (typically 1.5-3% of the input) were stained for CD11c and assayed by FACS.

Data analysis: Data was analyzed for statistical significance using Student's T test and a web-based calculator provided by Dr. Tom Kirkman, St. John's University (http://www.physics.csbsju.edu/stats/, accessed March/April 2004).

Acknowledgments

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Figure Legends:

Fig. 1: A) Ad5.GFP.γ F was grown in cells expressing the indicated fiber proteins, and viral particles analyzed for fiber with a monoclonal antibody against a conserved epitope in the fiber tail. As a loading control, the blot was reprobed for the viral penton base protein. **B)** Bone marrow-derived DC were infected with Ad5.GFP.γ F (100,000 particles/cell) with each of the indicated fibers. The percent of cells GFP-positive was determined 24 hours post-infection. '*' $p < .05$ vs. Ad5 fiber.

Fig. 2: A) Viruses with chimeric Ad5/16 or Ad5/37 fiber genes in place of the wt Ad5 gene. All Ads express EGFP from the CMV immediate/early promoter. **B**) Western blot of viral particles. Ad5.GFP.WT ('WT') and Ad5.GFP.F16 ('F16') are propagated in 293 cells. Ad5.GFP.F37 ('F37') is maintained in the Ad5 fiber-expressing 633 cells, so the resulting particles contain both the Ad5 and Ad37 fiber proteins. Virus for experiments is then produced by a single round of growth in 293 and contains only the virally-encoded Ad37 fiber. **C**) *In vitro*-generated DC were infected with 1000 or 10,000 particles/cell of Ad5.GFP.WT, Ad5.GFP.F16, or Ad5.GFP.F37 and the percentage of cells infected assayed by FACS. **D**) The mean fluorescence intensity of the cultures in (B) was determined. **E**) Cells were infected with the indicated Ads (10,000 particles/cell) and the percent survival determined 24 hours later. **F**) Cells were untreated, mock treated, or incubated with 10 mU/ml neuraminidase for one hour at 37 °C, infected with 10,000 particles/cell of Ad5.GFP.WT or Ad5.GFP.F37, and the percent of cells infected determined by FACS. Data are expressed as the percentage of infection in the untreated cells. '*': $p < .05$ vs. Ad5.GFP.WT (C, D) or vs. untreated cells (E, F).

Fig. 3: Mice (8/group) were injected subcutaneously with 1×10^8, 1×10^9, or 1×10^{10} particles of the indicated virus. Four weeks post-injection, splenocytes were cultured with synthetic peptides corresponding to the EGFP epitope HYLSTQSAL or the irrelevant ovalbumin sequence SIINFEKL and stained for surface CD8 and for intracellular IFN-γ production. The percentage of total CD8+ cells that are IFN-γ positive was calculated. A) Control experiments for assay specificity. Mice were injected with Ad5.GFP.WT (top row) or with the injection buffer alone

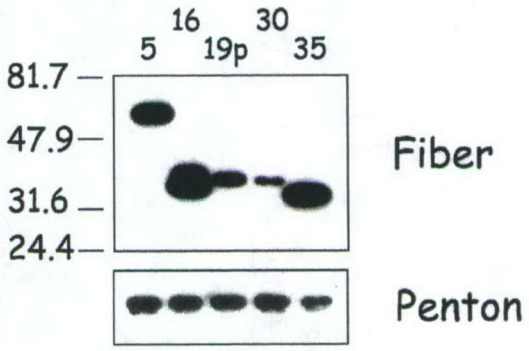
(bottom row), and incubated with the OVA (left panels) or EGFP (right panels) peptide. Anti-EGFP T cell reactivity is seen only in virus-injected animals. B) Splenocytes incubated with the EGFP peptide. C) Splenocytes incubated with the control peptide. Each point represents an individual mouse, and the means for each group are denoted by horizontal bars.

Fig. 4: Mice (four/group) were injected intravenously with 1×10^{11} particles of the indicated virus. Twenty-four hours post-injection, splenic DC were isolated with anti-CD11c magnetic beads (Miltenyi) and assayed for EGFP by FACS. Data presented is the percentage of $CD11c^{+}$ splenocytes that were also EGFP positive. '*': $p < .05$ vs. Ad5.GFP.WT.

Hsu et al. Fig. 1

A

Fiber	M_r
Ad5	62 kDa
Ad16	38 kDa
Ad19p	40 kDa
Ad30	41 kDa
Ad35	35 kDa



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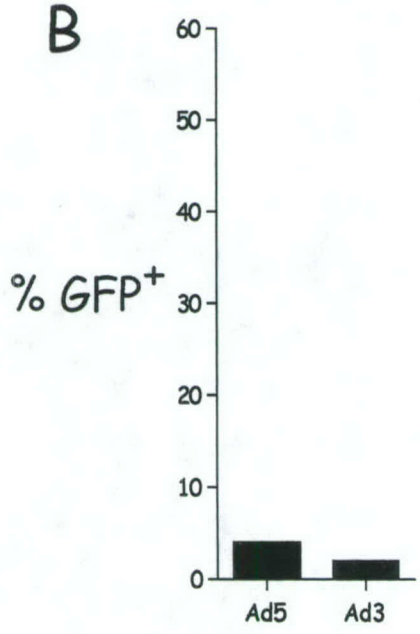
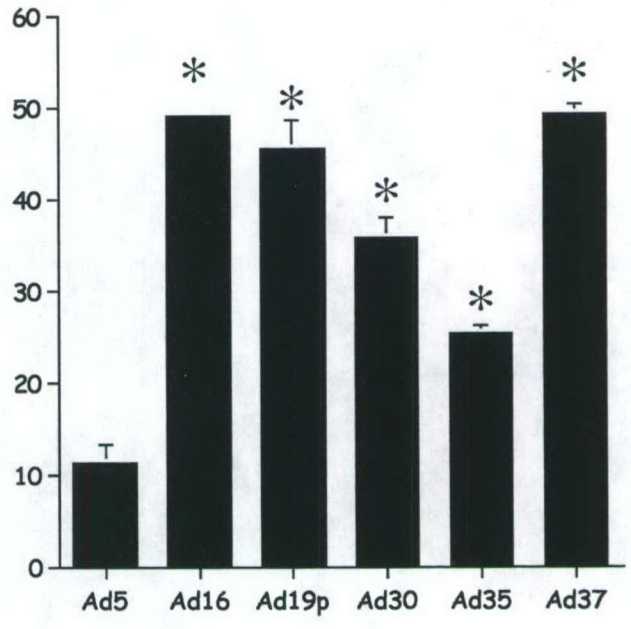
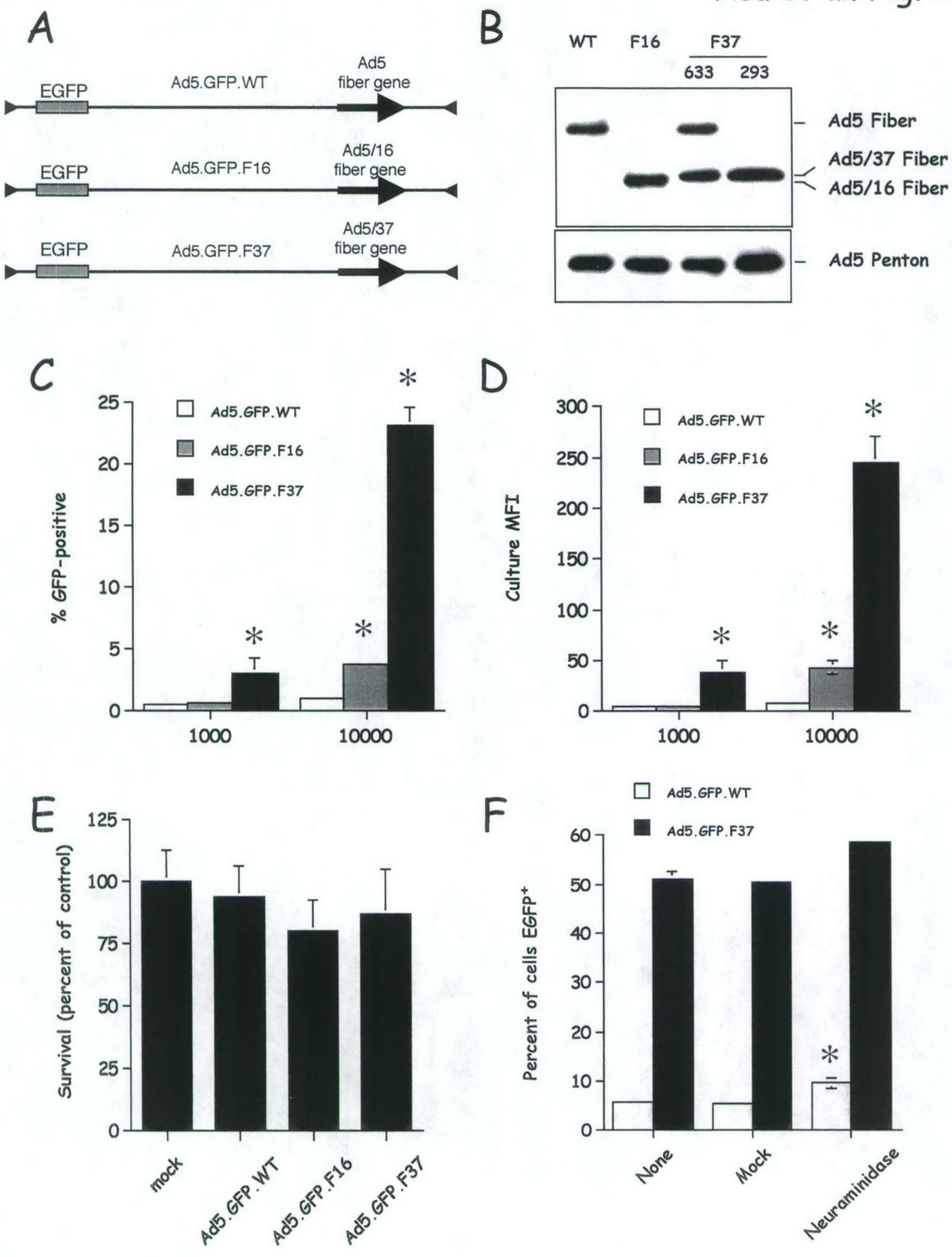


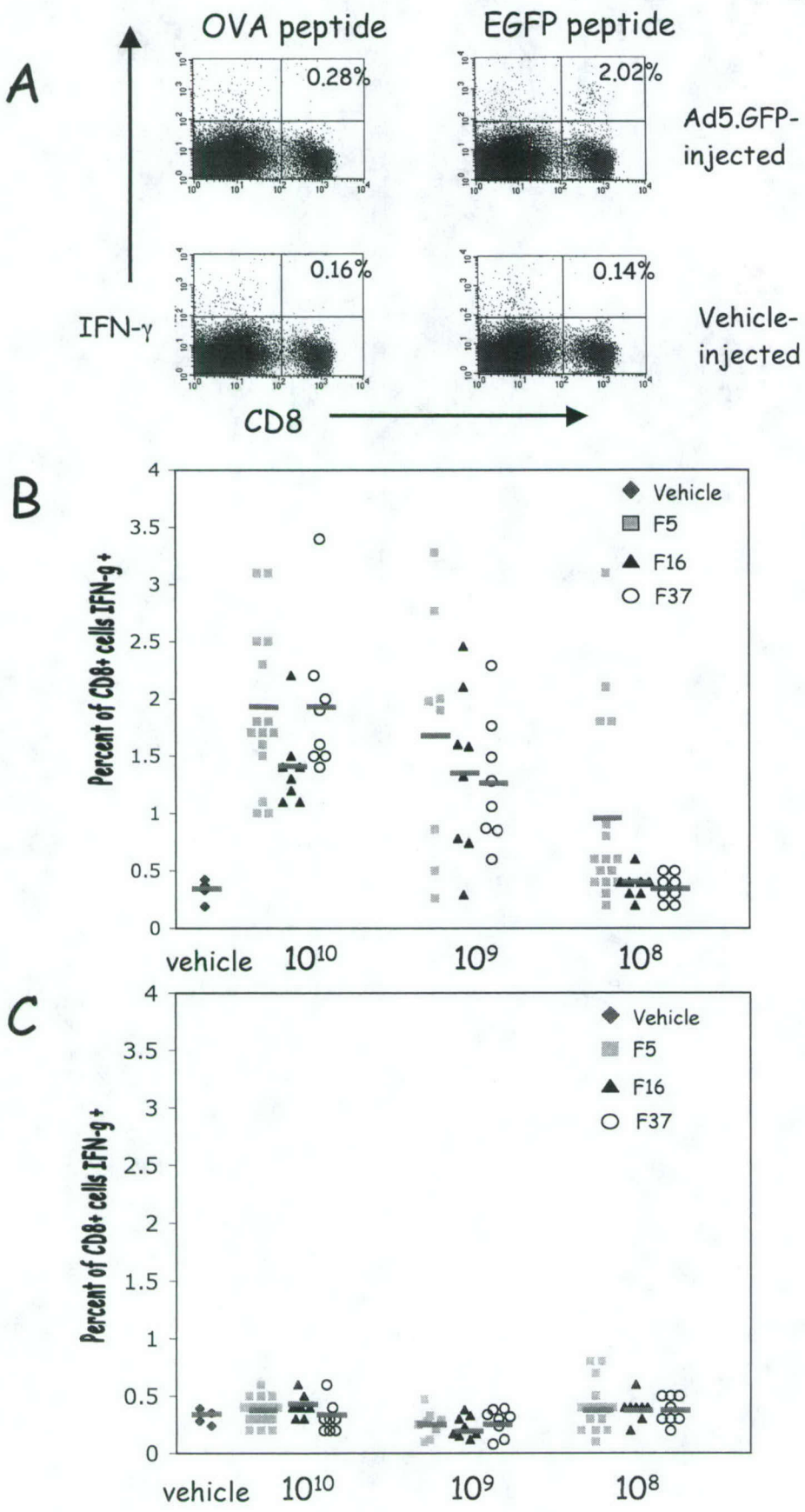


Fiber Protein on Particles

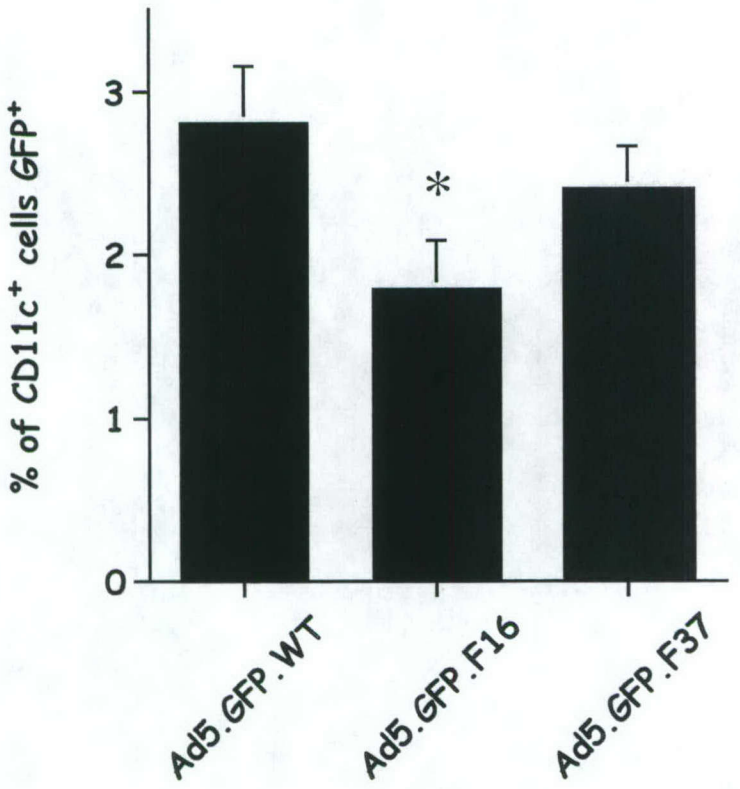
Hsu et al. Fig. 2



Hsu et al. Fig. 3



Hsu et al. Fig. 4



Adenoviral serotype 5 vectors pseudotyped with fibers from subgroup D show modified tropism *in vitro* and *in vivo*

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Abstract

Adenovirus (Ad5) serotype 5 vectors are commonly used for gene transfer. Pre-clinical studies have shown that their application to systemic gene delivery, however, is limited by their highly efficient uptake in the liver, principally mediated by receptor binding sites on the fiber shaft and knob domain. Targeting other sites *in vivo* using Ad requires vectors that lack hepatic tropism. We therefore sought to exploit Ad family diversity to isolate vectors that possessed poor hepatic tropism. We pseudotyped the fibers from Ad16 (subgroup B; Ad5/16), Ad19p (subgroup D; Ad5/19p) and Ad37 (subgroup D; Ad5/37) onto Ad 5 capsids and assessed infectivity profiles *in vitro* in multiple cell types and *in vivo* in rats. In rat, mouse and human hepatocytes, Ad5/19p and Ad5/37 both possessed a striking lack of hepatic cell infectivity compared to Ad5. Both vectors were, however, able to transduce human vascular endothelial and smooth muscle cells with efficiencies equal to or greater than non-modified Ad5. We evaluated liver uptake in 12-week old male rats following intravenous injection. In contrast to a vector with the wt Ad5 fiber, Ad5, both Ad5/19p and Ad5/37 produced significantly less virion accumulation (measured at 1h and 5 days) and transgene expression in the liver. Thus, Ad5/19p and Ad5/37 may be useful platforms for the development of targeted Ad vectors.

Keywords: gene therapy, viruses, liver, adenoviruses, real time PCR

Overview Summary

The use of Ad vectors for *in vivo* gene delivery targeted to sites other than the liver requires the construction of novel vectors that lack hepatic tropism. This can be achieved through mutation of Ad5 capsid proteins or pseudotyping fibers from other serotypes. Here we pseudotyped Ad5 vectors with fibers from serotype 19p and 37 and assess their infectivity *in vitro* and *in vivo*. *In vitro*, the infectivity of Ad5/19p and Ad5/37 for mouse, rat and human hepatocyte cells was dramatically reduced compared to non-modified Ad5 but both infected vascular cells with ease. Following systemic administration *in vivo*, Ad5/19p and Ad5/37 both showed reduced virion accumulation in liver, measured at 1h and 5 days post-injection leading to a substantial reduction in hepatic transgene expression. Hence, Ad5/19p and Ad5/37 are novel platform vectors for the future development of *in vivo* targeted Ad vectors.

Introduction

Recombinant adenovirus serotype 5 vectors (Ad5) are commonly used for gene delivery due to their broad cellular tropism *in vitro* and *in vivo* and their ability to infect dividing and non-dividing cells. Targeting of Ad5 as well as other viral vectors such as adeno-associated virus (AAV) is limited by their hepatic tropism (Huard *et al.*, 1995; Koeberl *et al.*, 1997). With respect to Ad5, the propensity for hepatocytes is extremely high since even local delivery of Ad5 to the arterial vessel wall leads to leakage resulting in vector dissemination and transduction of liver hepatocytes (Hiltunen *et al.*, 2000). Although Ad5 has been used extensively for pre-clinical and clinical localised gene delivery to the vasculature for vein grafts (George *et al.*, 2000) and in ischemia (Grines *et al.*, 2002; Hedman *et al.*, 2003), its use systemically is restricted to applications where genetic modification of the liver is not a disadvantage. This precludes the use of Ad5 as a vector to achieve site-specific gene delivery to target sites other than the liver unless detargeting strategies are employed. The tropism of Ad is dictated by many factors *in vivo* including primary and co-receptor usage, fiber length and rigidity. *In viv*o hepatic tropism of Ad5 is complex and involves rapid Kupffer cell-mediated clearance that limits hepatocyte transduction.

Targeting of Ad5 has been achieved using a plethora of techniques, broadly classified into capsid protein mutations, pseudotyping and antibody-mediated targeting [reviewed in (Nicklin and Baker, 2002)]. Mutations of amino acids involved in Ad5 binding to coxsackie and adenovirus receptor (CAR) and/or integrins have yielded varying results in mice, with both efficient liver detargeting (Einfeld *et al.*, 2001; Koizumi *et al.*, 2003) and no liver detargeting (Alemany and Curiel, 2001; Mizuguchi *et*

al., 2002; Martin *et al.*, 2003; Smith *et al.*, 2003b) reported. The reasons for the discrepancies in these studies are not yet clear. Recent evidence has provided further insight into Ad5's *in vivo* tropism for liver through binding to heparan sulphate proteogylcans (HSPG), putatively via a KKTK motif in the fiber shaft, mutation of which restricts liver transduction in mice and non-human primates (Smith *et al.*, 2003a; Smith *et al.*, 2003b). To date, however, only modest *in vivo* (systemic) retargeting of Ad5 vectors through capsid mutation and targeting peptide insertion has been reported (Reynolds *et al.*, 1999)(Nicklin et al., 2004).

Exploiting Ad serotype diversity is an attractive route to retarget Ad5 infectivity profiles *in vitro* and *in vivo* since fiber substitution will remove both the CAR and HSPG binding sites as well as conferring binding to alternate receptor(s) (Von Seggern *et al.*, 2000). At present there are 51 known human Ads subdivided into groups A-F. The tropism of Ad serotypes varies widely and indeed some have been exploited to achieve increased localised gene transfer to defined tissues, such as ocular cells (Von Seggern *et al.*, 2003), primary central nervous system cells (Chillon *et al.*, 1999) and vascular smooth muscle and endothelial cells (Havenga *et al.*, 2001; Su *et al.*, 2001; Havenga *et al.*, 2002). Systemically, detargeting liver in mice has been achieved using Ad5 vectors pseuodotyped with, for example, fibers from serotypes 3, 35, 40 and 41 (Sakurai *et al.*, 2003; Vigne *et al.*, 2003; Nakamura *et al.*, 2003; Nicol *et al.*, 2004; Schoggins et al., 2003).

Fibers from subgroup C viruses (such as Ad2 and Ad5) as well as viruses belonging to groups A, D, E and F have been shown to interact with soluble CAR, although this was measured under non-physiological conditions (Roelvink *et al.*, 1998).

Factors such as the length and rigidity of the fiber shaft, the charge of the knob domain, and the context of the fiber in the intact virion have all been shown to alter receptor usage and biodistribution *in vivo* (Shayakhmetov *et al.*, 2000; Wu *et al.*, 2003; Vigne *et al.*, 2003; Nakamura *et a*l., 2003). Many studies have focused on using subgroup B fibers, as these viruses do not interact with CAR (Havenga *et al.*, 2001; Miyazawa *et al.*, 2001; Schoggins *et al.*, 2003). Many subgroup B viruses (including Ad16 and Ad35) bind to CD46, a widely expressed complement regulatory molecule (Gaggar *et al.*, 2003). Less is known about subgroup D Ads and their infectivity *in vitro* and *in vivo*. Subgroup D viruses including Ad19a and Ad37 are associated with ocular and genital tract infections (Ford *et al.*, 1987). Of these, Ad37 has been studied in the most detail (Wu *et al.*, 2001; Arnberg *et al.*, 2002; Wu *et al.*, 2003). Ad37 can bind CAR in an overlay blot assay but does not utilise CAR for productive infection of cells, probably because the context of the fiber on the virion influences receptor binding . Sialic acid is a receptor for Ad37 (as well as Ad 8 and Ad 19a) and binding is hypothesized to be mediated through a charge interaction (Arnberg *et al.*, 2000). Recently CD46 has also been shown to be a target receptor for Ad37 in *in vitro* studies (Wu *et al.*, 2004). Biodistribution profiles *in vivo* and infectivity of primary cells has not, however, been assessed.

Here we substituted the fibers from Ad37 and the closely related Ad19p (subgroup D) onto Ad5-based virions and compared their transduction to non-modified Ad5 *in vitro* and *in vivo*. We found that Ad19p- and Ad37-pseudotyped vectors lack tropism for mouse, rat and human hepatocytes *in vitro* and demonstrate highly reduced uptake into liver following intravenous administration into rats.

Materials and Methods

Materials

Primary human saphenous vein endothelial (HSVEC), smooth muscle cells (HSVSMC) and HepG2 cells were cultured as described (Nicklin *et al.*, 2001a). Rat hepatocytes (ARL-6, obtained from ATCC) were cultured in Dulbecco's media, 20% serum and antibiotics. Mouse hepatocytes (CCL-2254, ATCC) were cultured in 1:1 F-12 Hams media, 10% serum, 5 μg/ml transferrin, 5 μg/ml insulin and 5 ng/ml selenium, 40 ng/ml dexamethasone with antibiotics. CHO (CD46 and control) cells were maintained in 1:1 F-12 Hams media, 10% serum, 1% G418 and antibiotics. CHO cells expressing human CAR were cultured in DMEM, 10% serum and antibiotics. Mouse endothelial cells SVEC 4-10 and IP-1B were maintained in Minimum Essential Media, 10% serum and antibiotics. Rat WKY endothelial cells (ECACC, UK) were maintained in complete endothelial media (TCS Cellworks, UK), 10% serum and antibiotics.

Pseudotyped adenovirus

Packaging cells expressing the Ad5, Ad5/3 and Ad5/37 fibers have been described (Wu *et al.*, 2001) (Von Seggern *et al.*, 2000). A cell line expressing the Ad19p fiber was constructed exactly as described for the Ad37 protein except that the fiber gene was amplified from Ad19p rather than Ad37 genomic DNA. For expression of the Ad16 fiber, the shaft and knob domains of the fiber gene were amplified using primers 5' CCG GTC TAC CCA TAT GAA GATG 3' and 5' TGG TGC GGC CGC TCA GTC ATC TTC TCT G 3', and cloned into pCDNA3.1/zeo(+) via *Not*I/*Nde*I sites (underlined),

creating pDV147. Addition of an *Nde*I fragment containing the Ad5 fiber tail domain and TPL fragment from pDV60 (Von Seggern *et al.*, 2000) was added to create the plasmid pDV156, which expresses a chimeric fiber protein with 17 amino acids of the Ad5 tail region fused to the Ad16 fiber shaft and knob. The fiber gene-deleted Ad5.βgal.ΔF (Von Seggern *et al.*, 1999) was propagated in packaging cell lines expressing the desired fiber protein. Viral particles (VP) were purified by CsCl ultracentrifugation and dialysed into 10 mM TRIS-pH 8.1, 150 mM NaCl and 10% glycerol. Viral particles were quantified by protein assay against BSA standards using the conversion 1 μg protein = 4 $x10^9$ VP. Genomes were quantified by real time PCR calculated from serial dilutions of equal VP of each virus amplified using *lac*Z primers and calculated against a standard curve. The following VP/genome ratios were obtained for each virus (in arbituary *lac*Z genome units/1.64 x 10^6 VP): Ad5/5 (0.83), Ad5/3 (0.65), Ad5/16 (0.71), Ad5/19p (0.88), Ad5/37 (0.72).

Cell Infections

Cells were plated out into 96 well plates 24h prior to infection. Cells were infected with 10,000 viral particles per cell (unless otherwise stated) in fresh media, incubated for 3 h at 37°C, washed twice in PBS, media replaced and maintained at 37°C until harvesting 72 h post infection. β-galactosidase was quantified using GalactoLight Plus (Tropix, USA). β-galactosidase activity was quantified by plate assay using a Wallac Victor 2 and recombinant β-galactosidase as standard. Protein concentrations were measured by BCA (Perbio, UK). All data are expressed as RLU/mg protein. CAR blocking experiments were performed using the CAR neutralising antibody (Cohen *et al.*, 2001)(a

kind gift from J. Bergelson) or control rabbit serum for 60 min at 4°C prior to addition of virus. CHO cells expressing CD46 and control (R-CHO) (a kind gift from Dr Atkinson). CHO cells expressing human CAR were a kind gift from Jeff Bergelson.

In vivo virus biodistribution

We used an established pre-dosing strategy (Tao *et al.*, 2001) to analyse *in vivo* vector biodistributions. This allows assessment of biodistribution in the absence of Kupffer-mediated clearance of Ad particles in vivo. The dosing regimen for rat was pre-optimised (Nicol *et al.*, 2004) from published studies in mice (Tao *et al.*, 2001). Briefly, $3x10^{11}$ VP of a null Ad (RAd 60) was injected into the femoral vein of 12 week-old male Wistar Kyoto (WKY) rats to reduce non-specific vector sequestration by saturating the Kupffer cells in the liver. 4 h post infusion $5x10^{10}$ VP of the pseudotyped vectors were *iv* administered. For virion tracking by real time PCR, DNA was extracted using the Qiagen DNA extraction kit (Qiagen, UK). DNA concentration in tissue samples was quantified using picogreen and analysed by Lightcycler (see below). For *in vivo* β-galactosidase expression, liver sections were fixed in 0.1% paraformaldehyde and stained with X-gal and photographed *en face*. Livers were embedded into paraffin and 5μm sections cut. Positive cells were counted in 3 fields of view of each of the 4 sections for each liver.

Real time polymerase chain reaction

A *lacZ* quantification standard curve was generated from serial dilutions of each Ad preparation using FastStart DNA master SYBR Green I (Roche, UK) (4mM $MgCl_2$) with

0.05 mM forward (5' ATC TGA CCA CCA GCC AAA TGG 3') and reverse (5' CAT CAG CAG GTG TAT CTG CCG 3') *lacZ* primers. 20 ng of total DNA from the liver or 10 ng from cell extracts were amplified and products quantified by melting curve analysis at crossover points. The following conditions were used: denaturation- 95 °C, 3 s; annealing- 60 °C, elongation- 72 °C, 20 s and data collection- 89 °C; 1 s (40 cycles).

Statistical analysis

In vitro experiments were performed in triplicate on at least three independent occasions. *In vivo* experiments were performed with at least 3 animals per group. Analysis was by unpaired Student's *t* test with statistical significance considered when $p<0.05$.

Results

Infectivity profiles *in vitro*

Our aim is to design and evaluate platform Ad vectors that are devoid of hepatic tropism *in vitro* and *in vivo* in order to construct vectors targeted for other sites for systemic *in vivo* gene therapy. We therefore first evaluated infectivity of Ad5/19p and Ad5/37 for human, rat and mouse hepatocytes *in vitro* and compared this to non-modified Ad5, Ad5/16 and Ad5/3 (Figure 1). Isn't this where we need to say 'hepatoma' instead of hepatocyte for thhe HepG2? We compared these to Ad vectors pseudotyped with fibers from the subgroup B serotypes 3 and 16, since these have previously been used for local and systemic gene delivery (Havenga *et al.*, 2001; Vigne *et al.*, 2003). Ad5, Ad5/3 and Ad5/16, but not Ad5/19p or Ad5/37, efficiently transduced human hepatocytes (Figure 1). While the transduction of hepatocytes appeared to be species selective for Ad5/3 and Ad5/16 (in that human cells but not those derived from rat or mouse were readily transduced), transduction by Ad5 was high in hepatocytes from all species (Figure 1). Ad5/19p and Ad5/37, however, showed dramatically reduced transduction in hepatocytes from all three species. For example, in human hepatocytes, levels of reporter gene expression were less than 1% of that evoked by Ad5 (Figure 1). We confirmed the reduced uptake of Ad5/19p and Ad5/37 compared to Ad5 into each cell type using real time PCR. Following exposure of cells to each virus for either 3 or 24 h at 37°C and immediate cell harvesting we observed significantly reduced virion DNA associated with cells for both Ad5/19p and Ad5/37 (Figure 2). Together, this demonstrates that both Ad5/19p and Ad5/37 have limited hepatocyte infectivity *in vitro*.

Ad37 can bind to both sialic acid (Arnberg *et al.*, 2002) and CD46 (Wu et al., in press). Although the Ad19p and Ad37 fibers only differ by seven amino acids (only two of which are in the knob domain), the Ad19p fiber does not bind sialic acid and therefore may target an alternate receptor(s) (Huang *et al.*, 1999; Wu *et al.*, 2001). We assessed the infectivity on two vascular cell types to define whether transduction was possible and whether differences between Ad5/19p and Ad5/37 could be found. We first transduced primary vascular EC isolated from human saphenous vein (Figure 3). Ad5/19p and Ad5/37 exhibited levels of transduction similar to Ad5 for HSVECs (Figure 3A). Interestingly, neither Ad5/19p nor Ad5/37 transduced primary human coronary artery endothelial cells (data not shown). This may reflect distribution patterns of the target receptor and further highlights the vascular heterogeneity. The Ad5/16 vector (as previously published, (Havenga *et al.*, 2001)) was able to transduce human vascular cell types at significantly higher levels than Ad 5 (Figure 3A) but was unable to transduce rat and mouse endothelial cells to high levels (Figure 3B+C). Ad5/19p and Ad5/37 showed higher levels of transduction in rat EC than Ad5/3 and Ad5/16 but remained lower than non-modified Ad5 (Figure 3B). None of the pseudotyped viruses showed any marked transduction in the two mouse EC lines evaluated (Figure 3C). This suggests that receptors for the Ad19p and Ad37 fibers are available for binding and can mediate transduction of endothelial cells from both human and rat. Ad5 transduction of vascular smooth muscle cells (SMC) is poor compared to other cell types both *in vitro* and *in vivo*. For example, in human and pig saphenous SMC high titers are required for transduction (George *et al.*, 2000) and transduction, at least in human venous SMC, occurs in a largely CAR-independent manner (Nicklin *et al.*, 2001b). We therefore assessed the capacity of

Ad5/19p and Ad5/37 to infect human saphenous vein SMC. Both vectors showed significantly improved transduction of HSVSMC compared to Ad5 although clearly lower than Ad5/16 (Figure 4).

Since Ad19p has been reported to interact with soluble CAR (Roelvink *et al.*, 1998) we investigated the potential role of CAR in the infection of HSVEC using an anti-CAR neutralising antibody (Cohen *et al.*, 2001) (Figure 5A). The antibody significantly reduced Ad5 transduction but did not affect Ad5/19p and Ad5/37 transduction. This was confirmed using CHO cells stably transduced with human CAR where Ad5 but not Ad5/19p or Ad5/37 efficiently transduced CHO-CAR cells (Figure 5B). Since CD46 has recently been shown to be a target receptor for Ad37 (Wu et al., in press) we assessed infectivity of CHO-CD46 cells (expressing the C2 form) (Oglesby *et al.*, 1991) with each virus (Figure 5C). As expected, Ad5/16 demonstrated high transduction of CHO-CD46 cells whereas Ad5 did not (Figure 5C). Ad5/19p did not transduce CHO-CD46 cells, whereas Ad5/37 showed a modest (2-5-fold) increase compared to CHO controls (Figure 5C). This indicates that interaction between the Ad37 fiber and this isoform of CD46 occurs but may be at a much lower affinity than that of serotype 16 fibers.

In vivo liver uptake

A principle finding of the *in vitro* infectivity profiles was the extremely low hepatic tropism of both Ad5/19p and Ad5/37, which suggests that they may be useful platform vectors from which to ultimately build systemically targeted vectors. We therefore assessed liver uptake of Ad5/19p and Ad5/37 following systemic administration and compared this to non-modified Ad5. We intravenously administered $5x10^{10}$ VP of each

virus into 12-week old WKY rats using the established pre-dosing regimen (Tao *et al.*, 2001). We used this procedure to allow evaluation of vector distribution in the absence of Kupffer cell interactions. Animals were sacrificed at 1 h or 5 days post infusion. At 1 h post infusion analysis of Ad5/19p and Ad5/37 produced a 63% and a 55% decrease, respectively in virion DNA accumulation in liver, compared to Ad 5 (Figure 6). Furthermore, at 5 days post-infusion only Ad5 virion DNA was detectable, confirming reduced virion sequestration in the liver for both Ad5/19p and Ad5/37 (Figure 6). Virus levels in other organs was low. In spleen, kidney, heart and thoracic aorta no differences were observed (data not shown). In lung, levels for Ad5/5 were also low but significantly reduced for Ad5/19p and Ad5/37 (data not shown). this is confusing. what about "Levels of Ad5 DNA in lung were low, but still significantly higher than those of Ad5/19p or Ad5/37." Levels in blood, sampled at 5 minutes and analysed by real time PCR for virion DNA were not different (not shown). At 1h, viruses were undetectable in blood (not shown). We quantified β-galactosidase production in transduced livers 5 days post-infection. Livers from Ad5-infused animals showed a high level of transduction observed by *en face* staining (Fig 7A). Virtually no staining was observed in the livers of either Ad5/19p or Ad5/37-infused animals (Figure 7A). Cell counts from liver sections stained with X-gal demonstrated 95±3% positive cells for Ad5, which was reduced to 12±2% for Ad5/19p and 7±1% for Ad5/37 (Figure 7B). Therefore, the reduced hepatic tropism for Ad5/19p and Ad5/37 observed *in vitro* translated to reduced virion accumulation and transgene expression in the liver following intravenous administration *in vivo*.

Discussion

The development of viral vectors that mediate site-specific *in vivo* gene delivery is becoming a reality through the use of antibody and peptide targeting systems. The identification and characterization of suitable platform vectors that lack hepatic tropism is an important aspect of this. Here we show that Ad5 vectors pseudotyped with the fibers from the subgroup D adenoviruses Ad19p and Ad37 lack hepatic tropism *in vitro* and *in vivo*. Both vectors were, however, capable of transducing saphenous vein endothelial and smooth muscle cells, suggesting that the receptor(s) for both Ad19p and Ad37 are present on at least these cell types.

Certain adenoviruses from subgroup D including Ad37, but not Ad19p, are associated with epidemic keratoconjunctivitis (EKC). Ad19p has only been isolated once in Saudi Arabia in 1955 and is not associated with any disease (Arnberg *et al.*, 1997). Therefore, the safety profile of Ad19p may be particularly favourable although further studies are required to address this. The fibers of Ad19p and Ad37 are thought to bind different primary receptors although they only differ by 7 amino acids (the knob domains differ by 2 amino acids) (Huang *et al.*, 1999; Wu *et al.*, 2001). Ad37 has been found to bind sialic acid (Arnberg *et al.*, 2002) as well as CD46 (Wu wt al., in press). Our *in vitro* studies confirm that CD46 may be used as a low affinity receptor for Ad5/37, but not Ad5/19p, although as there are several CD46 isoforms this will require further evaluation. Importantly, in these experiments we used the same C2 isoform-overexpressing cells utilized to identify CD46 as the receptor for group B viruses (Gaggar *et al.*, 2003). From the *in vitro* studies it is clear that receptor(s) for both Ad37 and Ad19p are present on both endothelial cells and SMC. Therefore, Ad5/19p and Ad5/37 may also find a niche

role for localised gene delivery to the vasculature thereby allowing equivalent or higher transduction than Ad5 but avoiding liver transduction if systemic dissemination occurs, as is the case for Ad5 (Hiltunen *et al.*, 2000). It will be important to isolate and characterise the receptors used by the Ad37 and Ad19p fibers.

Ad5/16 was superior to Ad5/19p and Ad5/37 at transducing human vascular cells, but was unable to transduce rat and mouse vascular cell types. Havenga *et al* (Havenga *et al.*, 2002) observed similar results with Ad16 being superior to Ad5 in human and rhesus monkey smooth muscle cells while pig, rat, rabbit, and mouse SMCs were less susceptible. They concluded that the receptor for Ad16 was not conserved between species. Since this paper was published it has been shown that CD46 is a receptor for Ad16 as well as many other subgroup B viruses (Gaggar *et al.*, 2003). In contrast to human CD46, which is expressed on many different cell types, expression of the murine protein is mainly restricted to testes (Inoue *et al.*, 2003). The fact that Ad5/19p and Ad5/37 can efficiently transduce rat vascular cells suggests that they may use another unidentified receptor in addition to CD46 (for Ad5/37), which is not expressed in murine liver.

This study highlights important species differences in Ad vectors that may complicate translating results from pre-clinical models into clinical trials. This problem has also been highlighted in Ad5 capsid mutation studies. Mutation of CAR and integrin binding has no effect on *in vivo* hepatic tropism in mice (Smith *et al.*, 2003b) but an identical batch of this virus completely detargets from rat liver (Nicol et al., submitted). It will therefore be important in future analyses of vector development to assess infectivity profiles in multiple species.

Andy-this part is still a little confusing, and i see why the reviewers don't like like it. Again the neutralizing Abs that are ALREADY present and therefore block administration are getting confused with Abs that get produced as a response to the virus (as Vigne et al. are talking about). What about we drop the discussion of fiber Abs completely (as you are eventually talking about making vectors that have the entire capsid swapped anyway)?

Neutralising antibodies are a potential problem for systemically injected Ad5 in humans. Derivation of vectors based entirely on Ad serotypes against which the human population has a low prevalence of neutralizing Abs is therefore an appealing approach to circumvent this issue. An Ad35-based approach has recently been described with transduction of target cells *in vivo* not compromised by pre-exisiting anti-Ad5 immunity (Vogels et al., 2003). Neutralising antibodies to Ad19p and Ad37 are present at a much lower frequency in the general population compared to Ad5, (Ad19p <20%, Ad37 <10%, Ad5 ~80%) (Vogels *et al.*, 2003), making these serotypes attractive candidates for use in humans.

In summary, we have identified suitable platform vectors for the development of targeted Ad vectors. Based on the infectivity profiles reported here, we propose their use for localised gene delivery to the vasculature and for the development of systemically administered, targeted therapies. For the latter, peptides capable of targeting chosen sites will require incorporation into Ad19p or Ad37 fibers.

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Figure legends

Figure 1: Infectivity of pseudotyped Ad vectors for hepatocytes cell lines. Human, rat and mouse hepatocytes cell lines were incubated with 10,000 viral particles per cell of the indicated virus for 3 h, washed and media replaced. After 72h the cells were lysed and β-galactosidase activity was quantified. *$p<0.01$ *vs* Ad5.

Figure 2: Virion attachment and internalisation in human HepG2 heptocytes. (A) HepG2 human hepatocytes were exposed to 10,000 viral particles/cell of the indicated virus for 3 or 24 h, the cell-associated DNA extracted and virion DNA quantified using real-time PCR. *$p<0.05$ vs Ad5. (B) Representative real time PCR trace.

Figure 3. Transduction profiles in vascular endothelial cells. (A) Human saphenous vein EC, (B) rat and (C) mouse ECs were incubated with 10,000 viral particles per cell of the indicated Ad for 3h, washed and media replaced. After 72h the cells were lysed and β-galactosidase activity was quantified. *$p<0.05$ vs Ad5. Representative stains for β-galactosidase are shown in (A) for human saphenous vein EC.

Figure 4. Transduction of human saphenous vein SMC. Human saphenous vein SMC were exposed to 10,000 viral particles/cell of the indicated virus and washed and media replaced. After 72h the cells were lysed and β-galactosidase activity was quantified. *$p<0.05$ vs Ad5.

Figure 5: Evaluation of CAR and CD46-dependent infectivity. (A) HSVEC were incubated with 20,000 viral particles per cell of either Ad5, Ad5/19p or Ad5/37 in the presence or absence of a CAR neutralising antibody. Normal rabbit serum was used as a control. After 72h the cells were lysed and β-galactosidase activity was quantified. $p<0.001$ *vs* Ad5 (B) CHO cells stably expressing hCAR were incubated with an increasing dose of pseudotyped vector for 3h. Cells were washed and media replaced. After 72h the cells were lysed and β-galactosidase activity was quantified. (C) Infectivity of each virus for CHO-CD46 cells *vs* control CHO cells.

Figure 6: *In vivo* liver sequestration of virions. $5x10^{10}$ viral particles of Ad5, Ad5/19p or Ad5/37 were infused *i.v* into rats 4h post using a pre-dose protocol (see Methods) and animals were sacrificed at either 1 h or 5 days, DNA extracted from liver and virion DNA quantified by real-time PCR. *$p<0.05$ *vs* Ad5. UN =undetectable.

Figure 7: Expression of transgene after *iv* administration. (A) *En face* photographs of liver sections stained for β-galactosidase activity with X-gal at 5 days post infusion with each Ad vector. (B) β-galactosidase positive cells were counted on 3 fields of view of 4 sections of liver for each Ad (n=4). *$p< 0.05$ *vs* Ad5.

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Membrane Cofactor Protein Is a Receptor for Adenoviruses Associated with Epidemic Keratoconjunctivitis†

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Subgroup D adenovirus (Ad) types 8, 19, and 37 (Ad8, -19, and -37, respectively) are causative agents of epidemic keratoconjunctivitis and genital tract infections. Previous studies showed that Ad37 binds to a 50-kDa membrane glycoprotein expressed on human ocular (conjunctival) cells. To identify and characterize the role of the 50-kDa glycoprotein in Ad37 infection, we partially purified this molecule from solubilized Chang C conjunctival cell membranes by using lentil lectin chromatography and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Liquid chromatography coupled to nano-electrospray ionization-tandem mass spectrometry was subsequently used to identify four Ad37 receptor candidates: CD46, CD87, CD98, and CD147. Immunodepletion analyses demonstrated that the 50-kDa protein is identical to CD46 (also known as membrane cofactor protein). The Ad37, but not Ad5, fiber knob bound to the extracellular domain of CD46, demonstrating a direct interaction of an Ad37 capsid protein with CD46. An antibody specific for the N-terminal 19 amino acids of CD46 also blocked Ad37 infection of human cervical carcinoma and conjunctival cells, indicating a requirement for CD46 in infection. Finally, expression of a 50-kDa isoform of human CD46 in a CD46-null cell line increased cell binding by wild-type Ad37 and gene delivery by an Ad vector pseudotyped with the Ad37 fiber, but not by a vector bearing the Ad5 fiber. Together, these studies demonstrate that CD46 serves as an attachment receptor for Ad37 and shed further light on the cell entry pathway of subgroup D Ads.

Adenoviruses (Ads) can infect many different human organs, including the upper respiratory system, the gastrointestinal tract, and the eye (for a review, see reference 32). The 51 known serotypes of human Ads are classified into six subgroups (A to F), defined by oncogenicity, erythrocyte hemagglutination patterns, and DNA homology (32). While subgroup B, C, D, and E Ads have been isolated from conjunctivitis (viral pink eye) patients, only certain subgroup D Ad serotypes (Ad8, Ad19, and Ad37) are associated with epidemic keratoconjunctivitis (EKC), a severe and highly contagious eye infection (17). EKC causes temporary blurred vision and irritation, with symptoms lasting weeks to months (42). In addition, Ad37 and the highly homologous Ad19 cause genital tract infections, such as cervicitis in women and urethritis in men, which are often accompanied by conjunctivitis (2, 21, 49). Currently, there are no effective treatments for these diseases. The receptor binding proteins (fibers) of Ad37 and Ad19a are identical (2, 40), suggesting that Ad37 and Ad19a tropism for the eye and genital tract is mediated by the expression of a common receptor.

Fibers of Ads from most subgroups bind to the coxsackievirus-Ad receptor (CAR) (8). Only subgroup B Ads (15, 19, 44, 46) and Ad37 (1, 52) have been definitively shown to use different cell attachment receptors. The fiber protein consists of three distinct domains: an N-terminal tail that attaches to the capsid, a central shaft that varies in length and flexibility, and a C-terminal knob that attaches to the receptor (for a review, see reference 13). The knobs of subgroup B Ads lack a binding site for CAR (9), but subgroup D Ads, including Ad37, have been shown to bind CAR immobilized on a solid support (44, 52), although with low affinity (28). The ability of Ad37 to bind CAR at the cell surface is further impaired by their relatively short (47) and rigid (12, 53) shaft domains, which prevent appropriate alignment with CAR on the cell plasma membrane. Thus, Ad37 likely binds to a different receptor on conjunctiva or the genital tract.

Ad37 has been reported to bind sialic acid carbohydrates presented on an unspecified glycoprotein (1) and/or unidentified 50- and 60-kDa glycoproteins expressed on conjunctival epithelial cells (52). On Chinese hamster ovary (CHO) cells, Ad37 recognizes α(2→3)-linked sialic acid displayed on a surface glycoprotein (1). A virus blot overlay protein blot assay (VOPBA) demonstrated calcium-dependent Ad37 binding to 50- and 60-kDa membrane glycoproteins expressed on permissive Chang C cells, a human conjunctival cell line (52). Although both glycoproteins are highly expressed on permissive Chang C cells, only the 60-kDa protein is expressed in less permissive A549 lung epithelial cells. Since the expression of the 50-kDa protein correlated with a substantial increase in Ad37 infection, we reasoned that this protein serves as the primary receptor for this virus type (52). In this study, we used a combination of biochemical techniques, immunological assays, and molecular biological approaches to identify the 50-kDa receptor and confirm its role in Ad37 infection. These studies increase our knowledge of virus tropism and lay the foundation for further studies to define the host cell factors that influence Ad infection of specific organ systems.

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MATERIALS AND METHODS

Cells and wild-type viruses. Human Chang C conjunctival cells, A549 lung epithelial cells, and HeLa cervical carcinoma cells were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). Cells were maintained in complete Dulbecco's modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, Calif.) with 10% fetal bovine serum (FBS). CHO cells that were stably transfected with an expression plasmid containing the C2 isoform of CD46 (CHO-C2 cells) (39) were maintained in complete DMEM–F-12 medium (Invitrogen) with 10% FBS and 0.5-mg/ml G418 sulfate (Invitrogen). CHO cells expressing human decay-accelerating factor (CHO-DAF) (33) were maintained in Ham's F-12 medium with 10% FBS. Wild-type Ad37 virus (ATCC) was propagated in A549 cells and purified by banding on cesium chloride (CsCl) gradients. Briefly, A549 cells were infected at 75 to 80% confluency with Ad37. Virions were purified 2 to 3 days postinfection by ultracentrifugation on 16 to 40% CsCl gradients and dialyzed into a mixture of 10 mM Tris (pH 8.1), 150 mM sodium chloride, and 10% glycerol. The viral protein concentration was determined by Bradford protein assay (Bio-Rad, Richmond, Calif.) and used to calculate the viral particle concentration (1 μg = 4×10^9 Ad2 virions).

Receptor purification and identification. The details of the Ad37 receptor purification and identification by mass spectrometry (MS) are described elsewhere (S. A. Trauger, E. Wu, S. B. Bark, G. Nemerow, and G. Siuzdak, submitted for publication). Briefly, approximately 5×10^8 Chang conjunctival cells were grown in a Cell Factory (Nunc, Roskilde, Denmark). The cells were washed twice with phosphate-buffered saline (PBS [pH 7.4]) and detached with 5 mM EDTA in PBS for 5 to 10 min. Detached cells were lysed by Dounce homogenization and fractionated by low- and high-speed centrifugation (52). The membrane proteins from the membrane (high-speed pellet) fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence and absence of 0.2 M β-mercaptoethanol and analyzed by VOPBA as previously described (52).

Membrane proteins, solubilized by the addition of CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}to 0.5%, were mixed with ~600 μl of washed, settled Sepharose beads cross-linked to lectin from *Lens culinaris* (lentil lectin Sepharose; Sigma, St. Louis, Mo.) and rocked for 1 h at 4°C. The entire volume was transferred into a disposable column (Bio-Rad) and drained. The lentil lectin Sepharose was washed with 8 ml of 20 mM Tris-HCl (pH 8.1), 0.5 M NaCl, 0.5% CHAPS (wash buffer). Bound proteins were eluted with 1.5 ml of 0.2 M α-methylmannoside, 1 mM EDTA, 16 mM Tris-HCl (pH 8.1), 0.4% CHAPS, and 0.4 M NaCl. All remaining bound proteins were eluted with 1.5 ml of 40 mM Tris-HCl (pH 8.1), 1% SDS, 10% glycerol, and trace bromophenol blue (1× SDS buffer). The virus-binding proteins present in each fraction were detected by an Ad37 VOPBA as previously described (52).

Proteins eluted with SDS buffer were concentrated and then subjected to preparative SDS-gel electrophoresis in an 8% Tris-glycine polyacrylamide gel (Invitrogen) without boiling or addition of reducing agents. The gel was stained with Simply Blue (Invitrogen), and a diffuse band from 48 to 50 kDa was excised. The proteins in the gel slice were deglycosylated with PNGase-F, digested with trypsin, reduced, and alkylated, and the resultant peptides were extracted with organic solvent. This complex mixture was analyzed by reverse-phase high-performance liquid chromatography coupled to nano-electrospray tandem MS (nanoLC-MS/MS). Masses of peptides and their fragment ions were compared to predicted masses obtained from proteins in the National Center for Biotechnology Information mammalian protein database to identify the proteins present in the gel slice.

Immunodepletion analysis. Two hundred microliters of soluble membrane proteins diluted with wash buffer was mixed with 20 μl of 0.5-mg/ml monoclonal antibody against CD46, CD87, CD98, or CD147 (BD Pharmingen, San Diego, Calif.) and rocked at 4°C for 2 h. Fifty microliters of protein A/G agarose (Pierce, Rockford, Ill.) was added to the mixtures, which were then rocked for another 2 h at 4°C. The agarose beads were gently pelleted, and the immunodepleted supernatant was removed and analyzed as described below. The beads were washed with 200 μl of wash buffer three times. Finally, 25 μl of 4× SDS buffer was added to the agarose bead pellets, and the beads were heated to 95°C for 5 min to elute the immunoprecipitated proteins.

Twenty microliters of each immunodepletion supernatant or 10 μl of the immunoprecipitation was separated by electrophoresis in an 8 to 16% Tris–glycine polyacrylamide gradient gel (Invitrogen). The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, Mass.). The membrane was blocked with 5% milk in a mixture of 10 mM Tris-HCl (pH 8.1), 150 mM NaCl, and 0.02% Tween 20 overnight at 4°C. Receptors were detected by an Ad37 VOPBA as previously described (52).

sCD46 and Ad fiber knob construction. The Ad37 fiber knob domain containing a T7 tag was expressed in bacteria and purified on a T7 tag affinity column as previously described (52). The Ad5 fiber knob containing a hexahistidine tag was expressed in bacteria in a similar fashion as previously described (54) and purified by Ni-agarose affinity chromatography (Qiagen) as described by the manufacturer's instructions. The extracellular domain of the C isoform of CD46 was amplified by a PCR from a mammalian expression plasmid coding for the C2 isoform of CD46 (39) by using Expand DNA polymerase (Roche) and 250 μM 5′ primer SCR1f (5′-GCTAGCTTGTGAGGAGCCACCAACATTTGA-3′) and 3′ primer EXTr (5′-GCGGCCGCATCCAAACTGTCAAGTATTCCT-3′). The PCR product was gel purified and cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was amplified from the culture of a colony of transformed cells and purified by using a Qiagen plasmid Miniprep kit according to instructions. Purified plasmid and mammalian expression plasmid pCEP-Pu-CHis (29) (kindly provided by O. Pertz, The Scripps Research Institute, La Jolla, Calif.) were digested with 5 U of NheI and NotI (New England Biolabs, Beverly, Mass.) restriction enzymes. Digested vector and insert were gel purified and ligated with T4 DNA Ligase (Invitrogen), and the resultant plasmid was then transformed into TOP10 cells (Invitrogen), amplified, and purified with the Qiagen plasmid Maxiprep. 293EBNA cells were transfected with the plasmid by using the calcium phosphate transfection kit (Invitrogen/Gibco). Secreted soluble CD46-C (sCD46) was purified from the culture media 3 to 4 days posttransfection by Ni-agarose affinity chromatography. Purified sCD46 was dialyzed against PBS and analyzed by SDS-PAGE and Western blotting.

Ad37 knob ELISA for binding. Wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Immulon 4 HBX; Dynex Technologies, Chantilly, Va.) were coated overnight with 1 μg of bovine serum albumin (BSA), purified Ad37 knob (52), or purified Ad5 knob. Wells were briefly blocked with Superblock in PBS (Pierce) and incubated with various amounts of sCD46 in BLOTTO in PBS (Pierce) containing 1 mM calcium chloride for 1.5 h at room temperature. Knob-coated wells were washed with PBS-$CaCl_2$, incubated with CD46 rabbit polyclonal antibody (Santa Cruz Biotechnology), washed, incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody, and washed again. The ELISA was developed with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrates and analyzed by measuring A_{405}.

Ad37 pseudotyping of an Ad5 vector by replacement of the fiber gene. To generate an Ad37 pseudotype with a low ratio of particles per infectious unit, the Ad5 fiber was removed from the Ad5 vector genome and replaced with the Ad37 fiber (Ad5.GFP.37f). First, a derivative of pAdEasy (22) with a chimeric Ad5/Ad37 fiber was generated. The plasmid pDV153 (D.Von Seggern, unpublished observations) contains the rightmost part of an E3-deleted Ad5 genome and has a unique MfeI site immediately downstream of the fiber stop codon. The 3′ fragment (encoding the shaft and knob domains) of the fiber gene was removed by digestion of pDV153 with SphI and MfeI. The corresponding portion of the Ad37 fiber was amplified from viral genomic DNA by using the PCR primers 5′-TACCAATGGCATGCTATCCCTCAAGG-3′ and 5′-AAACACGGCAATTGGTCTTTCATTC-3′ and inserted into MfeI/SphI-digested pDV153. (The SphI and MfeI sites are underlined.) The resulting plasmid (pDV154) contains a fusion protein consisting of 59 amino acids of the Ad5 fiber (the tail domain) and 306 amino acids (the shaft and knob domains) of the Ad37 fiber protein. The SpeI/PacI fragment of pDV154, containing the rightmost 6.2 kb of the Ad5 genome with the modified fiber, was then used to replace the corresponding fragment of pAdEasy1, creating pDV158. Recombination of pDV158 with pAdTrack in *Escherichia coli*, as described previously (22), generated a plasmid with a full-length Ad5 genome containing the chimeric fiber protein and a cytomegalovirus (CMV)-driven enhanced green fluorescent protein (eGFP) reporter gene cassette. This plasmid was transfected into 293 cells for virus production.

Infection assay. Human Chang C cells were detached with 5 mM EDTA in DMEM-FBS and split into aliquots of 40,000 cells per well in a 24-well plate. After culture at 37°C, cells were washed twice with sterile PBS to remove serum, and 125 μl of 2× medium 199 (Invitrogen) and 25 μl of 1 M HEPES (pH 7) were added to each well. Polyclonal antibodies raised against N-terminal peptides of CD46 and CD55 (Santa Cruz Biotechnology) were first dialyzed against PBS. Various amounts of antibodies were diluted to 80 μl in sterile PBS and added to cells for 1 h at room temperature with gentle rocking. Twenty microliters of 2.0×10^6 particles of Ad5.GFP.37f per μl was added to each well, and the wells were rocked at room temperature for 2 h. Virus was removed, and cells were cultured overnight in DMEM-FBS. HeLa cells were also preincubated with antibody or 1 mU of neuraminidase from *Vibrio cholerae* (Sigma) for 30 min at 37°C to increase enzyme activity. A total of 40,000 CHO-DAF and CHO-C2 cells were infected with 1,000 Ad5.GFP or Ad5.GFP.37f particles per cell for 1 h at 37°C in 250 μl

of 1:1 complete F-12 medium–PBS mixture with or without 25 μg of anti-CD46 antibody. The CHO cells were washed twice with complete F-12 medium and cultured overnight. The next day, human or CHO cells were detached with trypsin or 5 mM EDTA in complete medium and analyzed for GFP by flow cytometry (FACScan flow cytometer; Becton Dickinson, Franklin Lakes, N.J.).

Cell binding assay. To quantitate Ad binding, CHO-DAF and CHO-C2 cells were detached with 5 mM EDTA and washed twice with serum-free F-12 medium (SFF) to remove trace EDTA. A total of 2.5×10^5 cells were distributed to microcentrifuge tubes and pelleted by centrifugation. Some pellets were resuspended in 100 μl of SFF with 10 mU of neuraminidase (from *V. cholerae*; Roche). These cells were incubated at 37°C for 1 h and washed twice with SFF. A total of 10^9 wild-type Ad37 virions (4,000 particles per cell) were added to all samples in 100 μl of PBS in the presence or absence of 5 mM EDTA. Cells were rocked at 4°C for 1 h with virus. Cells were pelleted, washed twice with 200 μl of serum-free DMEM, and then resuspended in 200 μl of PBS. Total DNA (viral and genomic) was purified with the QiaAmp DNA Mini kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions.

Ten microliters of purified total DNA was mixed with 25 μl of TaqMan PCR master mix, rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents, 1.5 μl of 10 mM AQ1-D (5′-GCCACCGTGGGGTTCCTAAACTT-3′) and AQ2-D (5′-GCCGCAGTGGTCTTACATGCACATC-3′) primers (0.3 mM final), and 0.1 μl of 100 mM Adenoprobe (5′-6FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-TAMRA-3′; primers and reagents from Applied Biosystems, Foster City, Calif.). This mixture was then diluted to a final volume of 50 μl. These primers and Adenoprobe were designed to recognize the hexon gene of subgroup D Ads (23). Rodent GAPDH control reagents were designed to amplify a segment of the host cell genomic GAPDH gene. After initial denaturation and activation of the AmpliTaq Gold DNA polymerase by heating to 50°C for 2 min and then 95°C for 10 min, the amplicons were amplified with 40 cycles of 15 s at 95°C followed by 1 min at 60°C. Fluorescence of reporter dyes FAM and VIC was measured during each cycle in an ABI Prism 7900 sequence detection system (Applied Biosystems). Known amounts of wild-type Ad37 and purified cellular DNA were used as standards to determine the number of copies of Ad genomes and cell number in each sample.

Flow cytometry. CHO-DAF and CHO-C2 cells were detached by 5 mM EDTA in complete F-12 medium. Cells were incubated with medium (control) or a 1:200 dilution of mouse monoclonal antibodies against CD46 or DAF/CD55 (BD Pharmingen) for 1 h on ice, washed with medium, incubated with a 1:500 dilution of Alexa 488-conjugated antimouse antibody for 30 min on ice, and washed again. Cells were resuspended in PBS and analyzed for green fluorescence in a FACScan flow cytometer.

Statistical analyses. The effects of antibody treatment and concentration on infection of Chang C cells were separately analyzed by a two-way analysis of variance (ANOVA). sCD46 binding to BSA and Ad5 and Ad37 knobs was compared by general linear model repeated-measure analyses of all three data sets and each pair of data sets. The effects of treatments on Ad37 binding to CHO-C2 cells were analyzed by an ANOVA and separate *t* tests. Statistical analyses were performed by using SPSS 11.0 software (Chicago, Ill.).

RESULTS

Purification and identification of an Ad37 receptor. Chang C conjunctival cells, which express ~24,000 Ad37 binding sites per cell (24), were chosen as the source of the 50- and 60-kDa receptors. Approximately 10^9 Chang cells, carrying 2.4×10^{13} molecules (or 40 pmol) of receptor, were gently lysed by Dounce homogenization and fractionated by low- and high-speed centrifugation. The high-speed pellet was resuspended and then analyzed for the presence of virus binding proteins by using the VOPBA (Fig. 1A). As described previously, binding of Ad37 to the 50- and 60-kDa proteins was sensitive to chelation of calcium by EDTA (52). In addition, all three membrane proteins failed to bind Ad37 when treated with a reducing agent, suggesting the importance of inter- or intrachain disulfide bonds in receptor structure or function.

The diffuse pattern of the 50- and 60-kDa proteins suggested that these candidate receptors were glycosylated. Thus, we next purified the putative receptors further by using lentil lectin chromatography from detergent-solubilized Chang membrane proteins. This procedure separated these target molecules from >99% of the remaining membrane protein mass. The receptor fraction was concentrated and further separated by

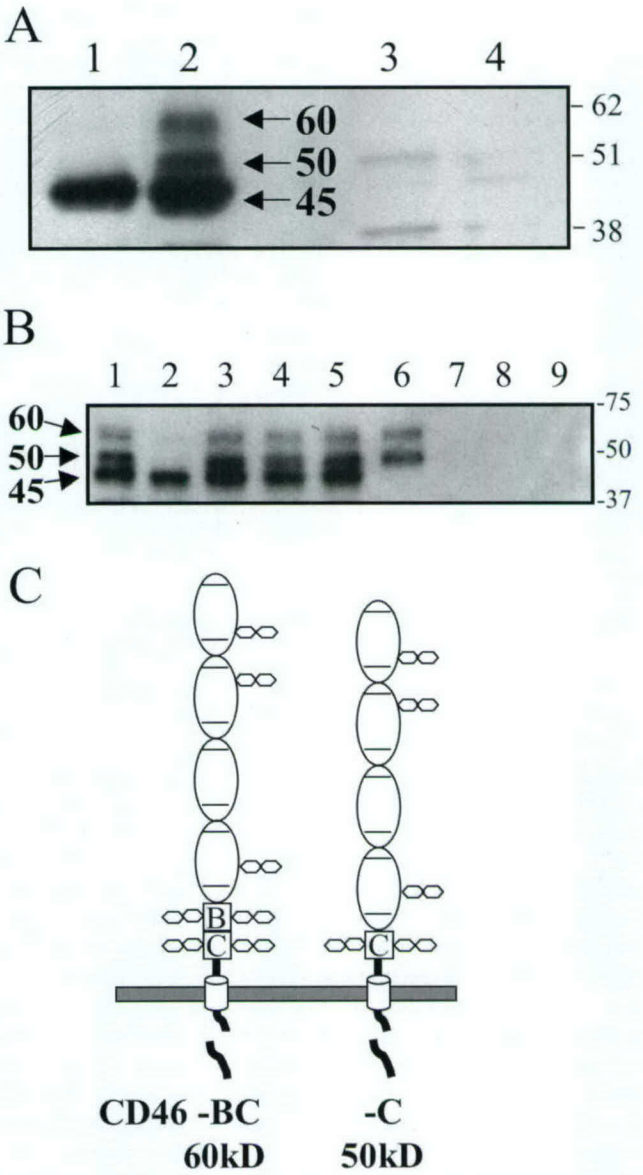

FIG. 1. Identification of the Ad37 receptor. (A) Membrane proteins from Chang C cells were probed on an Ad37-specific VOPBA in the presence (lanes 1 and 3) and absence (lanes 2 and 4) of 2 mM EDTA as described in Materials and Methods. Proteins in lanes 3 and 4 were reduced with β-mercaptoethanol prior to SDS-PAGE, while proteins in lanes 1 and 2 were not. The 45-, 50-, and 60-kDa proteins that bind Ad37 are indicated by arrows. (B) Soluble Chang membrane proteins (lane 1); proteins immunodepleted with antibodies directed against CD46, CD87, CD98, or CD147 (lanes 2 to 5, respectively); or proteins recovered in the immunoprecipitate (CD46, lane 6; CD87, lane 7; CD98, lane 8; CD147, lane 9) were probed with the Ad37 VOPBA. (C) The domain structure of CD46 is presented schematically. Horizontal lines in each SCR domain represent pairs of conserved disulfide bonds in the CD46 structure. Hexagonal chains represent locations of glycosylation. Four major isoforms (BC1, BC2, C1, and C2) are generated by alternative splicing of coding regions for serine/threonine/proline (STP)-rich domains (squares) and two possible cytoplasmic tails.

TABLE 1. Peptides identified by nanoLC-MS/MS analysis of the 50-kDa gel fragment

Putative receptor	No. of peptides observed	Sequence coverage (%)	Sequences of peptides identified
CD46	7	27	CEEPPTFEAMELIGK NHTWLPVSDDACYR GSVAIWSGKPPICEK VLCTPPPK GFYLDGSDTIVCDSNSTWDPPVPK GTYLTDETHR[a] ADGGAEYATYQTK[a]
CD87	5	22	ITSLTEVVCGLDLCNQGNSGR SGCNHPDLDVQYR GPMNQCLVATGTHEPK LWEEGEELELVEK VEECALGQDLCR
CD98	5	8	APAAEKEEAR SADGSAPAGEGEGV TLQR AAPAAEEKEEAR ALAAPAAEEKEEAR VQDAFAAAK
CD147	7	36	AAGTVFTTVEDLGSK GGVVLKEDALPGQK FFVSSSQGR KPEDVLDDDDAGSAPLK RKPEDVLDDDDAGSAPLK SELHIENLNMEADPGQYR PPVTDWAWYK

[a] From two cytoplasmic tails of CD46.

semi-native SDS-PAGE. Based on the fact that Ad37, but not Ad19p, recognizes the 50-kDa protein on Chang C cells, but not on less-susceptible A549 cells (52), the region of the gel corresponding to molecular masses of 48 to 50 kDa was excised and subjected to deglycosylation, reduction, and alkylation to improve peptide identification during MS analyses. Proteins were digested by trypsin, and subsequent analysis by reverse-phase liquid chromatography coupled to nanoLC-MS/MS matched peptides to over 50 known proteins, including endoplasmic reticulum and mitochondrial membrane proteins, actin, and keratins. However, only four plasma membrane proteins (CD46, CD87, CD98, and CD147) were detected (Table 1). These analyses are described in further detail elsewhere (S. A. Trauger et al., submitted for publication).

Immunodepletion analyses of candidate receptors. To determine if any of the four membrane proteins corresponded to the 50-kDa protein, antibodies specific for CD46, CD87, CD98, and CD147 were used to immunodeplete the 50-kDa protein in the Ad37 VOPBA (Fig. 1B). Antibodies against CD87, CD98, and CD147 failed to immunodeplete the 50- or 60-kDa virus-binding proteins (compare lanes 3, 4, and 5 with lane 1). In contrast, both the 50- and 60-kDa proteins were immunodepleted by the anti-CD46 antibody (lane 2). Moreover, the 50- and 60-kDa virus-binding molecules were present in the anti-CD46 immunoprecipitate (lane 6). The Ad37-binding 45-kDa protein (Fig. 1A), which served as an internal control, was identified as CAR by using a similar immunodepletion assay (data not shown). These data indicate that, of the four plasma membrane proteins identified by MS analysis, only CD46 directly interacts with Ad37.

CD46, also known as membrane cofactor protein (MCP), is an important regulator of complement activity (for a review, see reference 31). It protects host cells from destruction by the complement system by serving as a cofactor for the enzymatic degradation of C3b and C4b by serum factor I. CD46 also plays an important role in reproduction. The protein consists of a membrane signal peptide; four short consensus repeat (SCR) domains; one (C) or two (BC) regions that are rich in serine, threonine, and proline (STP-B and -C domains); a short sequence of unknown function; a transmembrane domain; and one of two cytoplasmic C-terminal tails. Four main isoforms (BC1, BC2, C1, and C2) are generated by alternative splicing (Fig. 1C). SDS-PAGE of CD46 (data not shown; (4) showed that the C and BC isoforms migrate to the same molecular masses as the candidate 50- and 60-kDa receptors for Ad37 (52).

Recombinant Ad37 fiber knob binds the extracellular domain of CD46. Attachment to cells by most Ads is mediated by the fiber protein (13, 38). Moreover, the cell receptor binding sites of several Ad serotypes have been located in the fiber knob domain (9, 18, 43, 44, 48). To investigate whether the Ad37 fiber knob is capable of direct binding to CD46, we produced soluble, recombinant CD46 (sCD46) containing the complete extracellular domain of the C isoform (Fig. 2A and B). The purified receptor was analyzed for direct binding to an Ad37 knob in an ELISA (Fig. 2C). Soluble CD46 was capable of binding to the Ad37 fiber knob ($P > 0.05$) but failed to recognize the Ad5 fiber knob ($P = 0.30$), as determined by comparison to nonspecific binding with BSA. These experiments confirm that Ad37 specifically associates with the extracellular domain of CD46.

Antibody to CD46 blocks Ad37 infection of Chang conjunctival and HeLa cervical carcinoma cells. To investigate whether CD46 is actually required for Ad37 infection of host cells, we analyzed virus infection in the presence of a CD46-specific antibody. Preincubation of Chang cells with a monospecific antibody directed against the N-terminal 19 amino acids of CD46 blocked Ad37 infection (Fig. 3A; $P < 0.001$) in a concentration-dependent manner (two-way ANOVA, $P < 0.001$). A control antibody specific for the N terminus of CD55 (DAF), another member of the complement control protein family (31), had no effect. Infection of HeLa cervical carcinoma cells, which also express the 50-kDa isoform of CD46 (39), was also inhibited by the anti-CD46 antibody (Fig. 3B). Although Arnberg et al. (3) previously reported that Ad37 uses sialic acid as a receptor on HeLa cells, we failed to detect an effect on Ad37 infection by treatment of these CD46-expressing cells with neuraminidase (Fig. 3B).

Expression of CD46 in CHO cells promotes Ad37 infection and cell binding. To further explore the function of CD46, we assessed infection of CHO cells expressing human DAF/CD55 (CHO-DAF; Fig. 4A) or the C2 isoform (50 kDa) of CD46 (CHO-C2; Fig. 4B) by an Ad vector pseudotyped with the Ad37 fiber. The extracellular domains of CD46 and CD55/DAF each contain four SCR domains and an STP-rich region. Expression of CD46 resulted in a four- to fivefold enhancement of Ad37 fiber-mediated gene delivery over expression of DAF/CD55 (Fig. 4C). The relatively low level of Ad infection of CHO-DAF cells conferred by the Ad37 fiber was similar to the level observed with receptor-null CHO cells (data not

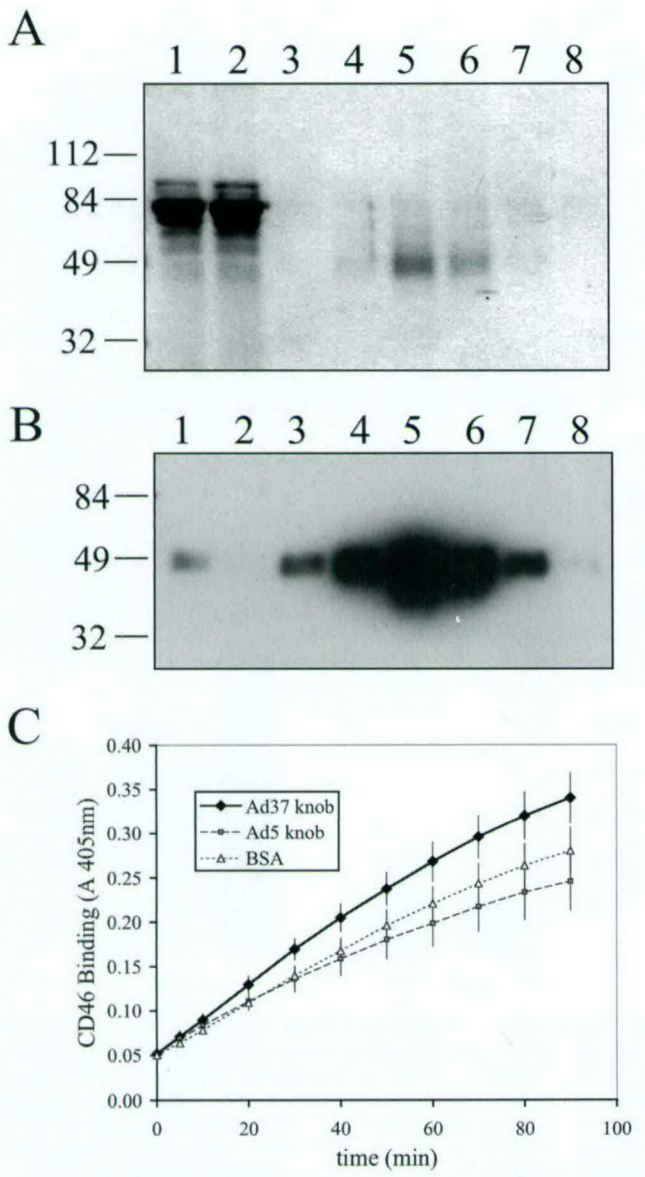

FIG. 2. Purification and analysis of sCD46 binding to Ad fiber knobs. The extracellular domain of the C isoform of CD46 with a C-terminal hexahistidine tag was purified from 293EBNA cell culture medium by Ni-agarose affinity chromatography. Culture medium (lane 1), column flowthrough (lane 2), wash fraction (lane 3), and elution fractions 1 to 5 (lanes 4 to 8, respectively) were analyzed by SDS-PAGE (A) and anti-CD46 Western blotting (B). Binding of purified sCD46 to immoblized BSA or Ad37 or Ad5 fiber knobs in an ELISA (C). Data represent the averages and standard deviations of quadruplicate determinations.

shown) and consistent with previous reports (1). In contrast to Ad37, CD46 expression did not improve Ad5-mediated gene delivery. Antibody directed against CD46 also abolished Ad37 infection of CD46-expressing cells, confirming the requirement for this receptor in Ad37 infection. These data indicate that CD46 specifically promotes Ad infection via the Ad37 fiber, but not the Ad5 fiber.

Ads use separate receptors for attachment (8) and internalization (51). Cell binding is primarily mediated by the fiber protein, while internalization is enhanced by the interaction between the Ad penton and α_v integrins for Ad serotypes in subgroups A to E (35, 51). Monoclonal antibodies against $\alpha_v\beta3$ and $\alpha_v\beta5$ also blocked pseudotyped Ad37 infection (12), suggesting that the interaction between the Ad37 fiber and its receptor mediates virus attachment, not internalization. To determine if CD46 serves as an attachment receptor, we measured Ad37 binding to receptor-null CHO-DAF and receptor-expressing CHO-C2 cells. The expression of CD46 increased Ad37 binding to CHO cells approximately fivefold in comparison to CHO-DAF cells (Fig. 4D). The increased binding correlates well with the four- to fivefold increase in gene delivery due to CD46 expression (Fig. 4C), indicating that the susceptibility

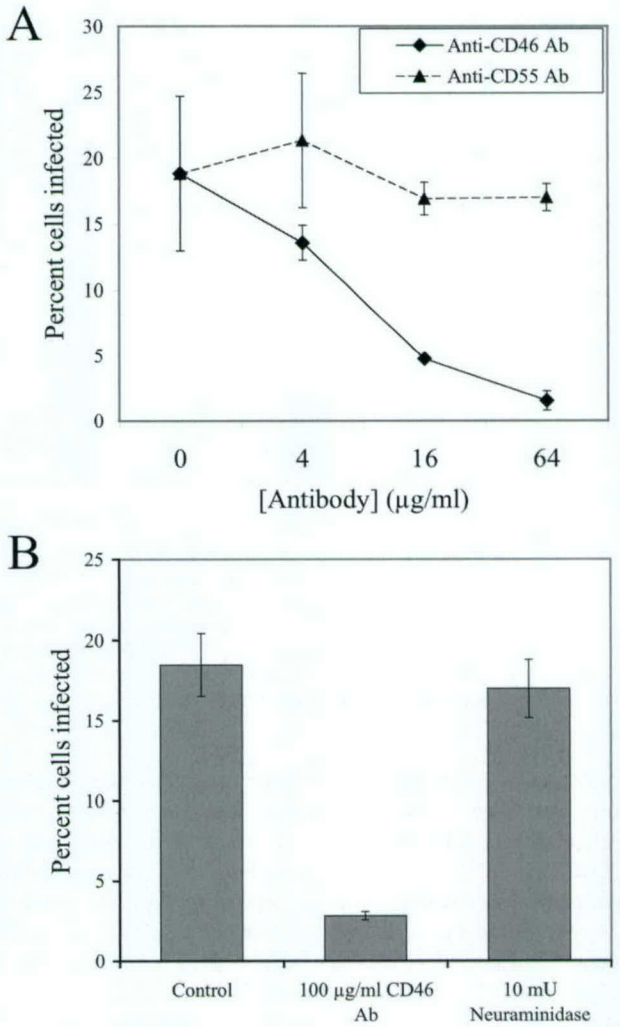

FIG. 3. Antibody specific for CD46 blocks Ad37 infection of Chang conjunctival and HeLa cervical epithelial cells. Chang conjunctival cells were preincubated with various concentrations of an antibody (Ab) specific for the N-terminal 19 amino acids of CD46 or CD55 (DAF) and then infected with 1,000 particles per cell of Ad vector pseudotyped with the Ad37 fiber (A). HeLa cells were preincubated with 100-μg/ml CD46 antibody or 1 mM *V. cholerae* neuraminidase prior to infection with Ad37 vector (B). Infection was measured as expression of GFP transgene by flow cytometry. Data represent the averages and standard deviations of triplicates.

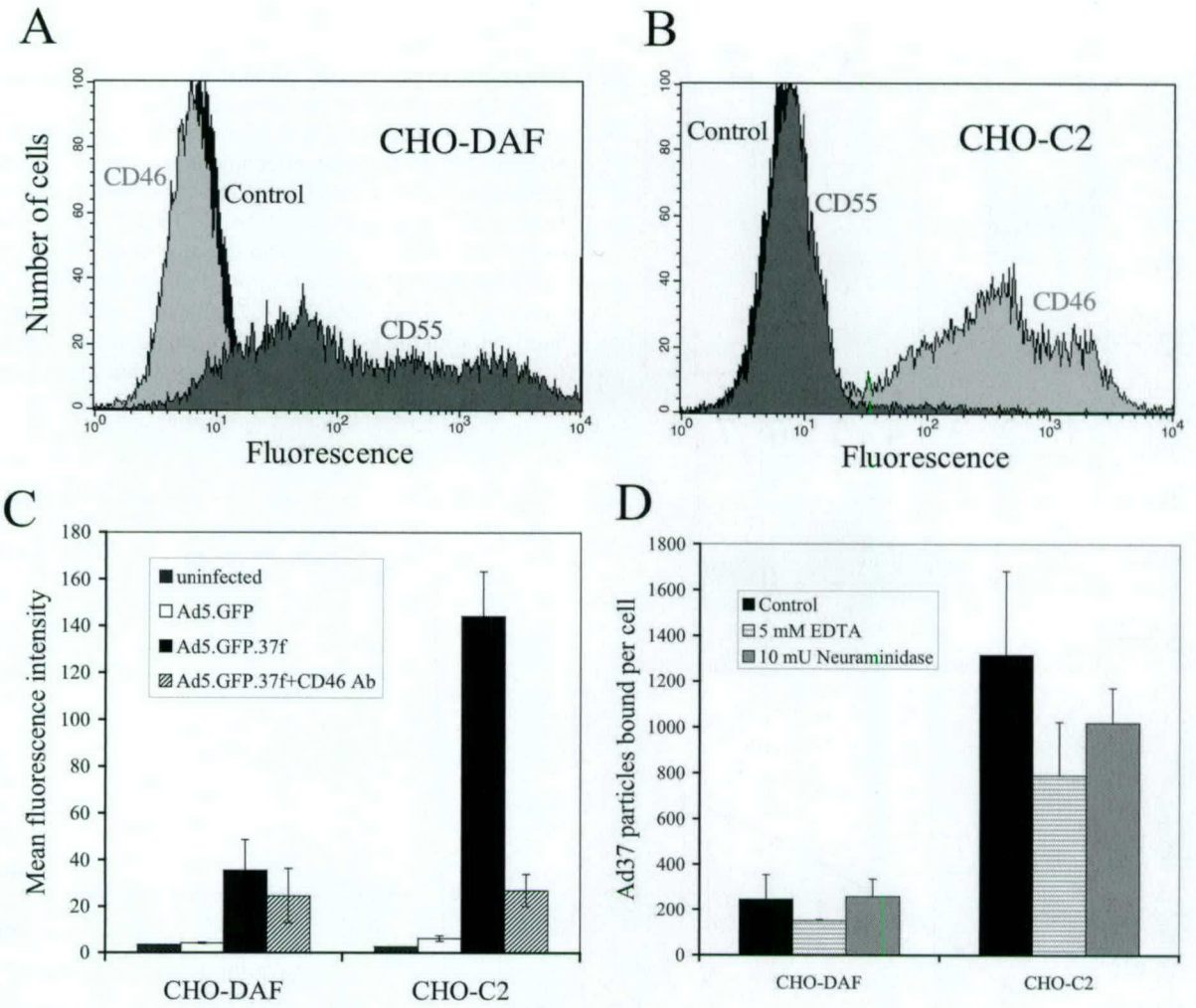

FIG. 4. CD46 expression selectively promotes Ad37 infection. CHO cells expressing the human DAF (CHO-DAF) (A) or the C2 isoform of CD46 (CHO-C2) (B) were analyzed by flow cytometry for CD46 (dark gray) and CD55 (light gray) expression with monoclonal antibodies. CHO-C2 cells or CHO-DAF cells were infected with Ad vectors carrying the fibers of Ad5 or Ad37 in the absence or presence of 100 μg of anti-CD46 antibody (Ab) per ml (C). GFP transgene expression was measured 24 h postinfection by flow cytometry. Data represent the averages and standard deviations of quadruplicates. CHO-DAF and CHO-C2 cells were incubated with wild-type Ad37 virus particles at 4°C for 1 h to measure virus binding to cells (D). Bound virus particles and cell counts were determined by quantitative PCR. Data represent the averages and standard deviations of triplicate or quadruplicate determinations of virus counts divided by cell counts (number of particles bound per cell).

conferred by CD46 expression is mainly due to Ad37 binding. Removal of cell surface sialic acid with neuraminidase had little effect on Ad37 binding to CHO cells expressing DAF or CD46 ($P = 0.20$), indicating that sialic acid is not required for binding to these cell types. In agreement with previous studies (24), we found that chelation of divalent cations led to a decrease in virus binding ($P = 0.07$), suggesting a role for divalent cations in Ad37 adhesion to the cell. Together, these data demonstrate that CD46 functions as an attachment receptor for Ad37.

DISCUSSION

Ad37 is a causative agent for EKC and genital tract infections. In an effort to understand Ad37 entry and pathogenesis, we applied improvements in MS and bioinformatics to identify a 50-kDa Ad37 binding protein as CD46. In contrast to previous receptor identification efforts, this approach did not require pure protein. This technique allowed the identification of proteins in a complex sample and substantially reduced the number of possible receptors from thousands of molecules in the human genome to a manageable four candidates. Immunodepletion of each of these four candidates indicated that the Ad37 capsid selectively binds to CD46. We showed that the Ad37 fiber knob directly binds to the extracellular domain of CD46 and that this interaction requires no other viral or cellular proteins. Chelation of divalent cations also had a negative effect on virus adhesion to the cell surface of CD46-expressing CHO cells, which is consistent with the calcium dependence of Ad37 adhesion to human cells (52). The crystal structure of CD46 revealed the presence of a calcium ion located between SCR1 and SCR2, which was important for crystallization (11). This calcium ion may help stabilize CD46 in an orientation that allows efficient Ad37 association. Ablation of Ad37 binding to CD46 upon reduction (Fig. 1A) can be attributed to the eight

disulfide bonds that stabilize the four SCRs of CD46 (Fig. 1C). This finding suggests that the Ad37 binding site is located in the SCR domains of CD46. In support of this possibility, Ad37 binding and infection of cells were significantly reduced by an antibody raised against the N-terminal 19 residues of CD46 in SCR1. Importantly, expression of CD46 in CHO cells promotes Ad37, but not Ad5, infection. Together, these studies indicate that CD46 serves as an attachment receptor for Ad37.

CD46 was also recently identified as the receptor for subgroup B Ads, Ad11 and Ad35 (19, 46). In contrast to our studies, Segerman et al. (46) found that an antibody specific for SCR3 and/or -4, but not an antibody specific for SCR1, blocked Ad11 binding to CHO cells expressing the BC1 isoform of CD46 and partially inhibited Ad11 binding to A549 cells (46), suggesting that Ad11 binds an epitope in SCR3 or -4. Unlike Ad37 adhesion to cells (52), Ad11 association with CD46 did not require divalent cations (46). Moreover, expression of the C2 isoform of CD46 on CHO cells had little, if any, effect on gene delivery by Ad9, another subgroup D member (19). In conjunction with the identification of the Ad11 and Ad35 receptor, our experiments indicate that members of the same virus family use distinct modes of interaction to bind to the same receptor and that members of the same Ad subgroup can use different receptors. Further studies will be necessary to more precisely map the sites of Ad37, Ad35, and Ad11 binding to CD46.

In addition to its role as a receptor for Ad37, Ad35, and Ad11, CD46 is also a receptor for several other microbial pathogens, including *Neisseria gonorrhoeae* (27), human herpesvirus 6 (HHV-6) (45), and measles virus (16, 36). *N. gonorrhoeae* and measles virus are also associated with conjunctivitis (20, 37), and HHV-6 causes corneal infections and retinitis in AIDS patients (41). In addition, both Ad37 and *N. gonorrhoeae* are causative agents for genital tract infections. An interesting link between these ocular and genital tract infections is the common receptor usurped by diverse microbes. Moreover, CD46 ligation has been shown to downregulate certain cellular immune responses (34), some of which arise following signal transduction via the receptor cytoplasmic domain (50). Obviously, these receptor-mediated events could influence host responses to these pathogens and thereby impact disease progression. The use of an immunologically important molecule by a growing number of microbial agents therefore may not be coincidental but rather may have evolutionary benefits.

The expression of the C isoforms of CD46 in conjunctival (Chang C) and cervical (HeLa) cells (39) explains the in vitro tropism of Ad37 for these cell types. CD46 is selectively expressed in both the anterior (corneal epithelium) and posterior (retina) chambers of the eye (10) and has also been found in ocular fluids (14). It is likely that the presence of CD46 in the eye contributes to Ad37 tropism for ocular tissue. The identification of CD46 as an Ad37 receptor does not completely explain the limited tropism of this virus, however, because this receptor is broadly distributed on many cell types. One possible explanation is that certain CD46 isoforms (i.e., C versus BC) abundantly expressed in the eye or genital tract may be preferentially used by Ad8, Ad19, and Ad37. In other words, the selective expression of particular CD46 isoforms, which has been shown in the brain and central nervous system (26), may further limit Ad37 tropism in vivo. Ad37 binds well to Chang conjunctival cells, which express both BC and C isoforms of CD46, but poorly to A549 lung epithelials cells (24), which only express the BC isoform (data not shown). This directly correlates with earlier observations that Ad37 only bound the 60-kDa membrane protein on A549 cells, but bound both 50- and 60-kDa proteins on Chang cells (52). Only the expression of the 50-kDa isoform correlates with Ad37 binding and infection, suggesting that Ad37 preferentially associates with the C isoforms of CD46 on cell surfaces. In addition, expression of the BC1 and BC2 isoforms of CD46 on CHO cells resulted in only a small (20 to 30%) increase in Ad37 binding compared to binding of CHO cells (46), indicating that the BC isoforms mediate Ad37 binding poorly. In contrast, we show that expression of the C2 isoform of CD46 on CHO cells increases Ad37 binding and infection of CHO cells by over 300% (Fig. 4), indicating that Ad37 can use the C2 isoform of CD46 as a cell receptor. The contributions of the cytoplasmic tails (CYT1 and CYT2) to Ad37 infection are unknown.

The extracellular domain of the C isoforms differ from the BC isoforms only in the absence of the 15-residue STP-B domain, which has been shown to be heavily O-glycosylated and sialylated. Desialylation with neuraminidase decreased the apparent molecular mass of the BC isoform by ~8 kDa and changed its pI from 5 to 7 (5). The effect of STP-B on CD46 structure and function is unclear, although it has been shown that the BC isoforms bind C4b and protect cells against complement cytotoxicity better than C isoforms (30). Cofactor activity for C4b cleavage, which maps to SCR2 and SCR3 (25), is apparently affected by STP-B, despite there being no known direct interaction between C4b and STP-B. *N. gonorrhoeae*, which appears to bind SCR3 of CD46 (27), also preferentially binds to the BC isoforms of CD46 (27). Thus, Ad37, like C4b and *N. gonorrhoeae*, may recognize a specific CD46 isoform, but in this case prefers the C isoforms of CD46. Ad37 attachment to cells has been shown to be sensitive to the orientation of molecules at the cell surface due to its short and rigid Ad37 fiber protein (53). One possibility is that the heavily sialylated STP-B domain could alter the orientation of CD46 with respect to the cell surface and negatively affect the presentation of the Ad37 binding site at the cell surface.

We observed that Ad37 infected receptor-null CHO cells better than Ad5 (Fig. 4C), a finding previously attributed to sialic acid binding by Ad37 (1). However, the typically weak nature of protein-carbohydrate interactions is inconsistent with the nanomolar dissociation constants (K_D) of Ad37 binding to human cells: K_D = 3.5 nM for fiber (24) and 0.35 nM for virions (3). It is possible that sialic acid carbohydrates serve as low-affinity virus binding sites on some rodent cells, although rodents are not known to be vectors for Ad37. However, we did not observe a specific requirement for sialic acid for Ad37 binding of CHO cells (Fig. 4D) or Ad37 infection of Chang conjunctival cells (52) or HeLa cells (Fig. 3B). Ad37 VOPBA of neuraminidase-treated Chang conjunctival cells showed that Ad37 no longer bound desialylated 60-kDa protein but still bound the 50-kDa protein (52). Upon further inspection of the VOPBA after identifying the Ad37 receptor as CD46, we found that the 60-kDa protein (CD46-BC) had instead decreased in apparent molecular mass by ~8 kDa to slightly above that of the 50-kDa protein (CD46-C). The 50-kDa band

also decreased in molecular mass by ~3 kDa, similar to previous analyses of MCP with glycosidases (5), and was hidden by merging with the 45-kDa CAR band. This result clearly demonstrated that wild-type Ad37 virions retained the ability to bind desialylated CD46.

Another possibility is that sialic acid on molecules other than CD46 serves as a coreceptor to strengthen Ad37 interaction with cells. Strengthening of virus-receptor interaction via the use of sialic acid has been reported for reovirus serotype 3 (T3) adhesion to the reovirus receptor (7). Binding of sialic acid by reovirus T3 affected the kinetics of adhesion to HeLa cells but did not change the affinity. Reovirus, which attaches to cells via a fiber-like protein called σ1, still requires the presence of its protein receptor, junction adhesion molecule, for efficient binding to cell surfaces (6). Reovirus binding to sialic acid induces apoptosis in the infected cell. It is unknown whether Ad37 binding to sialic acid has a similar effect or contributes to disease symptoms, but sialic acid binding does not appear to be essential for virus entry into cells expressing CD46.

The identification of the receptor for Ad37 sheds further light on the host cell factors that facilitate Ad cell attachment and entry. MCP/CD46-fiber interaction may also represent a potential new target for treatment of Ad-associated EKC and genital tract infections. Antiviral agents that interfere with the Ad37 fiber-CD46 association may delay or prevent the dissemination of this highly infectious eye disease in hospitals and eye clinics.

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ARTICLE

In Vivo Transduction of Photoreceptors or Ciliary Body by Intravitreal Injection of Pseudotyped Adenoviral Vectors

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Strategies for retargeting adenoviral (Ad) vectors have been developed, but their *in vivo* efficacy remains to be demonstrated. Gene delivery to specific ocular cell types represents an approach to treating many diseases that cause irreversible blindness. One of these cell types, the photoreceptor (PR), is not infected by standard Ad5-based vectors. We evaluated gene delivery after intraocular injection of Ads pseudotyped with three different fiber proteins and found three distinct patterns of infection. An intravitreally injected Ad5 vector readily infected the iris, corneal endothelium, and ciliary body, while few cells in the retina expressed transgene product. In contrast, an Ad3-pseudotyped virus selectively transduced ciliary body, of interest for treating diseases such as glaucoma. A vector pseudotyped with the fiber protein of Ad37 transduced PRs as well as ciliary body. This finding has potential application to the treatment of retinal degenerative or neovascular diseases. These studies demonstrate cell type-selective gene delivery *in vivo* with retargeted Ads, provide information about the cellular tropisms of several Ad serotypes, and should lead to improved strategies for preserving vision.

Key Words: adenovirus, pseudotype, tropism, photoreceptor, retina, *in vivo*, ocular, gene transfer, intravitreal, targeting

INTRODUCTION

Most gene therapy studies to date have used viral vectors in order to take advantage of the virus' highly evolved mechanisms for cell entry. Although the majority of experiments have relied on native viral tropisms, recent work has demonstrated that the targeting of viral vectors, including adeno-associated virus (AAV), lentivirus, and adenovirus (Ad), can be altered either by modifying the capsids or by pseudotyping [1–11]. Far fewer studies have addressed the issue of targeting distinct cell populations *in vivo* [7,9–12].

Ad-based vectors have several desirable properties, including the capacity to transfer large genes and the ability to readily generate high titers of stable particles. Ad tropism is largely determined by binding of the viral fiber protein to its specific cell-surface receptor [13]. The 51 human Ad serotypes fall into six subgroups and use a number of different fiber receptors. The vectors in common use are based on Ad5 (subgroup C) or the nearly identical Ad2, both of which bind to cells by means of the coxsackievirus and adenovirus receptor (CAR) [13]. Internalization of virus is then mediated by interaction of the viral penton base protein with α_v integrins [13]. CAR is widely expressed, but a number of clinically relevant cell types lack CAR and are resistant to Ad5 infection. CAR's widespread expression also makes it likely that many nontarget cells will be infected. Because binding to cells is distinct from internalization, vector pseudotyping with different or modified fibers can change receptor usage and result in altered cell targeting [1–4,6,14–16]. A large number of other Ad serotypes exist in nature, which should allow infection of distinct cell populations by means of their differing tropisms. However, there is almost no information available on the identity or distribution of the receptors used by most of these.

One cell type not readily infected by Ad5 is the retinal photoreceptor (PR) [17–20]. This is the primary cell responsible for vision through its function as a phototransducer, capturing photons of light and transducing them into neural signals. When PRs malfunction or degenerate,

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vision is severely compromised. It is estimated that 1 in 3500 individuals in the United States suffer from one of many degenerative diseases of PRs, a group of pigmented retinopathies collectively called retinitis pigmentosa. Although there currently is no treatment or cure for these diseases, the past few years have seen important advances that provide clues to their etiology. The genetic defects underlying these conditions [21–23] are often in genes expressed specifically in PR or in the closely associated retinal pigment epithelium (RPE), making these cells candidates for targeted treatments. Although phenotypic correction of retinal degenerative disease by gene therapy has been demonstrated in animal models [19,20,24–30], translating this concept to broadly useful clinical therapies will require improved gene delivery tools. Several vector systems have been used for ocular gene delivery, but no single system currently has all the properties needed for widespread clinical use. Whereas RPE is readily transduced by Ad, AAV, or lentiviral vectors, PR transduction has been more difficult. Long-term transgene expression in PRs has been reported after subretinal injection of AAV or lentivirus-based vectors [28,31–33]. More recent work has shown that PR infection can be improved by pseudotyping AAV vectors with the AAV5 capsid [10,11], although the retargeted vectors retain a somewhat limited capacity for inserted DNA. Ad vectors that efficiently infected PRs would allow transfer of large transcriptional units or of large cDNAs such as the 7.1-kb *ABCR* gene involved in Stargardt disease, which as the most common recessive hereditary macular dystrophy affects ~1 in 10,000 individuals [22,23].

The absence of PR infection by Ad5 suggests that this cell type does not express CAR, and therefore the use of a different fiber receptor might permit transduction. Fiber proteins from a number of different Ad serotypes have been used in vector pseudotyping and have been found to alter viral tropism [1–5,14,16]. Ad3 and Ad7 (subgroup B) use an unidentified receptor distinct from CAR and appear (at least *in vitro*) to have a tropism broader than that of Ad5 [1,3]. Their *in vivo* tropism, in particular their ability to infect the various ocular cell types (including PR), has not yet been evaluated. Tropism of subgroup D viruses is even less well understood. Some group D Ads, including the related serotypes Ad19a and Ad37, are associated with epidemic keratoconjunctivitis [34]. Ad19a and Ad37 have identical fiber genes and use the same fiber receptor [35], which is known to be distinct from CAR [5,36] or from that used by Ad3 and Ad7.

In an effort to target specific ocular cell types, we have identified cells in the eye that are preferentially infected by Ad vectors pseudotyped with fiber proteins from Ad5, Ad3, or Ad37. We observed distinct patterns of infection using each fiber protein, and found that PR cells were transduced by a vector with the Ad37 fiber. This work demonstrates that retargeted Ad vectors can infect distinct cell types within a target organ *in vivo,* and can selectively deliver reporter genes to ocular cell types associated with retinal degenerative and glaucomatous disease.

RESULTS

Ocular Transduction Using CAR or the Ad3 Receptor

We initially compared the ocular cell types that could be infected by means of CAR or the Ad3 receptor, using a pair of viruses that are identical except for their fiber genes. Av1LacZ4 and Av9LacZ4 [3] are first-generation vectors that include genes encoding the wild-type Ad5 fiber or a chimeric fiber with the Ad3 receptor-binding domain, respectively (Fig. 1A). Both carry a nuclear-targeted β-galactosidase reporter gene driven by the Rous sarcoma virus (RSV) promoter, to allow identification of infected cells.

Virus was injected (~3 μl at 1×10^9 vector particles/μl) into the vitreous cavities of adult Balb/C mice. Although it is difficult to compare infectious titers of viruses that use different receptors, Av1LacZ4 and Av9LacZ4 have been shown to have roughly equivalent ratios of particles to plaque-forming units [3]. Globes were enucleated 7 days after injection, then fixed and stained to identify cells expressing β-galactosidase. As shown in Figures 2A and 2B, injection of virus particles (Av1LacZ4) with the Ad5 fiber resulted in strong reporter gene expression in many regions of the eye. Staining was especially prominent in iris and ciliary body, with scattered positive cells in the neural retina, lens epithelium, and corneal endothelium. Evaluation of the retinal staining showed that most of the neuronal cells infected were in the inner retina (ganglion cell layer) and that few, if any, were in the PR layer (Fig. 2C). A few cells in the RPE were also positive. These results agree closely with previous reports of a broad Ad5 ocular tropism, notable for the absence of PR infection [17–19,27,37].

Considering our previous work, we anticipated that the Ad3-targeted Av9LacZ4 would infect a wider variety of cells [1,3]. Surprisingly, infection was more selective, with β-galactosidase expression largely confined to the ciliary body (Figs. 2D and 2E). Scattered staining was also observed in the iris and inner retina. However, as with the Ad5-targeted virus, transgene expression was not observed in PRs (Fig. 2F). Because the two viruses are identical except for their fiber loci, the different patterns of transduction are most likely due to differential expression of the respective fiber receptors (see Discussion). The data shown in Figure 2 are representative of three independent experiments and a total of 24 eyes injected with each vector.

Infection of Photoreceptors Using the Ad37 Fiber

The foregoing results indicate that Ads using CAR or the Ad3 receptor transduce distinct cell types *in vivo,* but we did not observe PR transduction in either case. Because Ad37's receptor is distinct from CAR or the Ad3 receptor,

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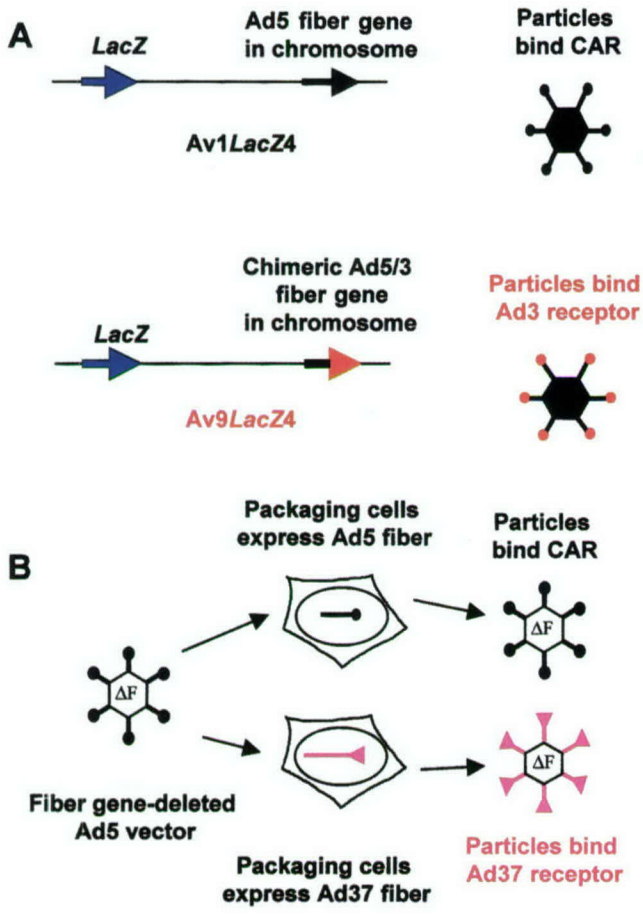

FIG. 1. Viruses used in these studies. (A) Av1LacZ4 and Av9LacZ4 are Ad5-based vectors containing a β-galactosidase transgene and are identical except at the fiber locus. Av9LacZ4 contains the receptor-binding domain of the Ad3 fiber and therefore infects cells by means of the Ad3 receptor rather than CAR. (B) Generation of Ad5 particles pseudotyped with fibers of different serotypes. The fiber-deleted vector Ad5.GFP.ΔF is grown in cells expressing either the Ad5 or Ad37 fiber-protein. The resulting particles (designated Ad5.GFP.ΔF/5F or Ad5.GFP.ΔF/37F) contain only the fiber expressed by the cells and have the corresponding tropisms.

we also evaluated the Ad37 fiber for its use in ocular gene delivery. We earlier described a pseudotyping system in which a fiber gene-deleted Ad5 vector is retargeted by fiber complementation in cells expressing the fiber protein of interest (Fig. 1B) [1]. Particles of the fiber-deleted vector Ad5.GFP.ΔF containing the Ad5 or Ad37 fiber proteins (Ad5.GFP.ΔF/5F or Ad5.GFP.ΔF/37F, respectively) were generated by growth in packaging cell lines expressing the relevant fibers. Ad5.GFP.ΔF carries an enhanced green fluorescent protein (EGFP) transgene driven by the cytomegalovirus (CMV) immediate-early promoter, which facilitates image analysis in infected cells. The viral particles produced in these cells have been shown to use CAR or the Ad37 receptor, respectively, but have not previously been evaluated for their *in vivo* tropism [1,5].

Ad5.GFP.ΔF/37F or Ad5.GFP.ΔF/5F (~3 μl of viral suspension at 1×10^8 particles/μl) was injected intravitreally. As in the experiments just described, we injected equal numbers of viral particles rather than attempting to use equal infectious doses of viruses using different receptors. Animals were euthanized 6 days after injection and their eyes examined for EGFP fluorescence. Examination of ocular sections showed that the EGFP signal in Ad5.GFP.ΔF/5F-injected eyes was primarily localized to the corneal endothelium, iris, ciliary body, lens epithelium, and a few cells of the anterior retina, in agreement with the experiments just described as well as with previous findings that Ad5 does not infect PRs (data not shown).

In contrast to the results obtained with Ad5.GFP.ΔF/5F injection, we did observe fluorescence of cells in the posterior retina in a substantial number of the Ad5.GFP.ΔF/37F-injected eyes (Fig. 3A). To identify PRs unambiguously, retinal sections were stained with an opsin antibody, which labels the PR outer segments. Co-localization of the opsin (red) and EGFP (green) signals in the outer segments confirmed that the $EGFP^+$ cells were indeed PRs (Figs. 3C–E). The soluble EGFP was distributed throughout the cells, allowing visualization of the characteristic PR shape (outer segment, inner segment, and the region surrounding the nucleus are clearly visible) (Fig. 3F). We also observed some areas of very bright fluorescence just posterior to the PR. In some cases, this signal could be localized to the RPE layer (Fig. 3G), but much appeared to be in the intercellular space between PRs and RPE (see Discussion). Although the PR was the major cell type transduced by Ad37-pseudotyped virus, we also detected EGFP in ciliary body, trabecular meshwork, and scattered cells throughout the iris (Fig. 4A) as well as occasional Müller cells (Fig. 4B). In several independent experiments, we observed PR transduction in 27% of the Ad5.GFP.ΔF/37F-injected eyes, but in none of the eyes injected with Ad5.GFP.ΔF/5F (summarized in Table 1). The difference in PR infection was highly significant ($P = .01$, Fisher's exact test). To ensure that the PR transduction we observed after intravitreal injection was not due to the virus gaining access to the subretinal space by misinjection or tears introduced during the injection process, Evans Blue dye was injected intravitreally using the same technique and apparatus used to inject the virus. Examination of sections taken 4 hours after infection showed that the dye was present throughout the interior of the globe, but did not reach the subretinal space (data not shown).

Discussion

After many years of investigation, there are surprisingly little data on *in vivo* Ad tropism, even for the widely studied Ad2 and Ad5. Almost nothing is known about infection by other Ads, despite increasing interest in their

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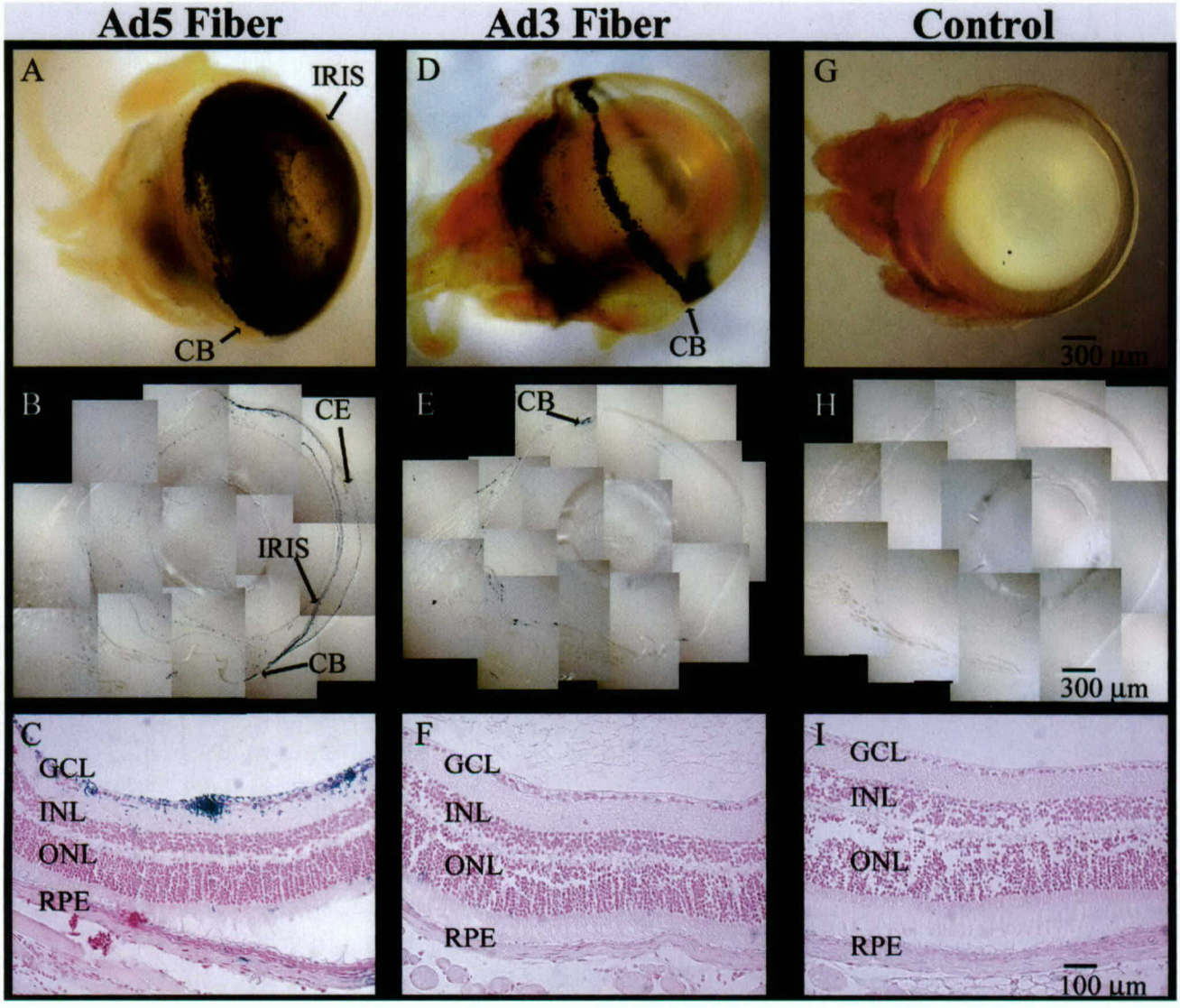

FIG. 2. Eyes from Balb/C mice injected intravitreally with Av1LacZ4 (Ad5 fiber) or Av9LacZ4 (Ad3 fiber) and stained for nuclear β-galactosidase expression. (A) and (B) Whole eye and section, respectively, following injection of Av1LacZ4. Note the prominent staining in the iris, corneal endothelium (CE), and ciliary body (CB). (C) section of an Av1-injected retina stained with X-gal and counterstained with hematoxylin. Most of the observed staining is in the ganglion cell layer (GCL) and inner nuclear layer (INL), whereas few, if any, PR nuclei in the outer nuclear layer (ONL) are positive. (D–F) Av9LacZ4-injected eye, stained as in panels (A–C). Staining is predominantly limited to the ciliary body (CB), with more scattered positive cells in the iris and retina. (G–I) Control (PBS-injected) eye, treated as in panels (A–C).

use as vectors. The pseudotyping system allows us to study the distribution of alternate receptors such as Ad3 and Ad37 *in vivo,* and the eye offers an opportunity to study viral infection of different cell types such as epithelium, endothelium, and many specialized neuronal cell types in a single, contained organ. The pattern of ocular cell infection has been described for Ad5 [17–19,27,37] but not other serotypes. We detected three distinct patterns of transduction by Ads equipped with three different fibers. Using a vector with the Ad37 fiber, we show for the first time that Ad can transduce PRs. More generally, this study demonstrates the concept of using retargeted Ads to reach specific cell types of interest *in vivo.*

Standard Ad5 vectors fail to transduce PRs, probably because these cells lack either CAR or α_v integrins. Because both our Ad5- and Ad37-pseudotyped vectors use α_v integrins [38,39], PR infection by the Ad37-typed virus is likely due to use of an alternate fiber receptor. Both a 50-kDa molecule expressed on Chang C epithelial cells and an unidentified sialic acid-containing protein have

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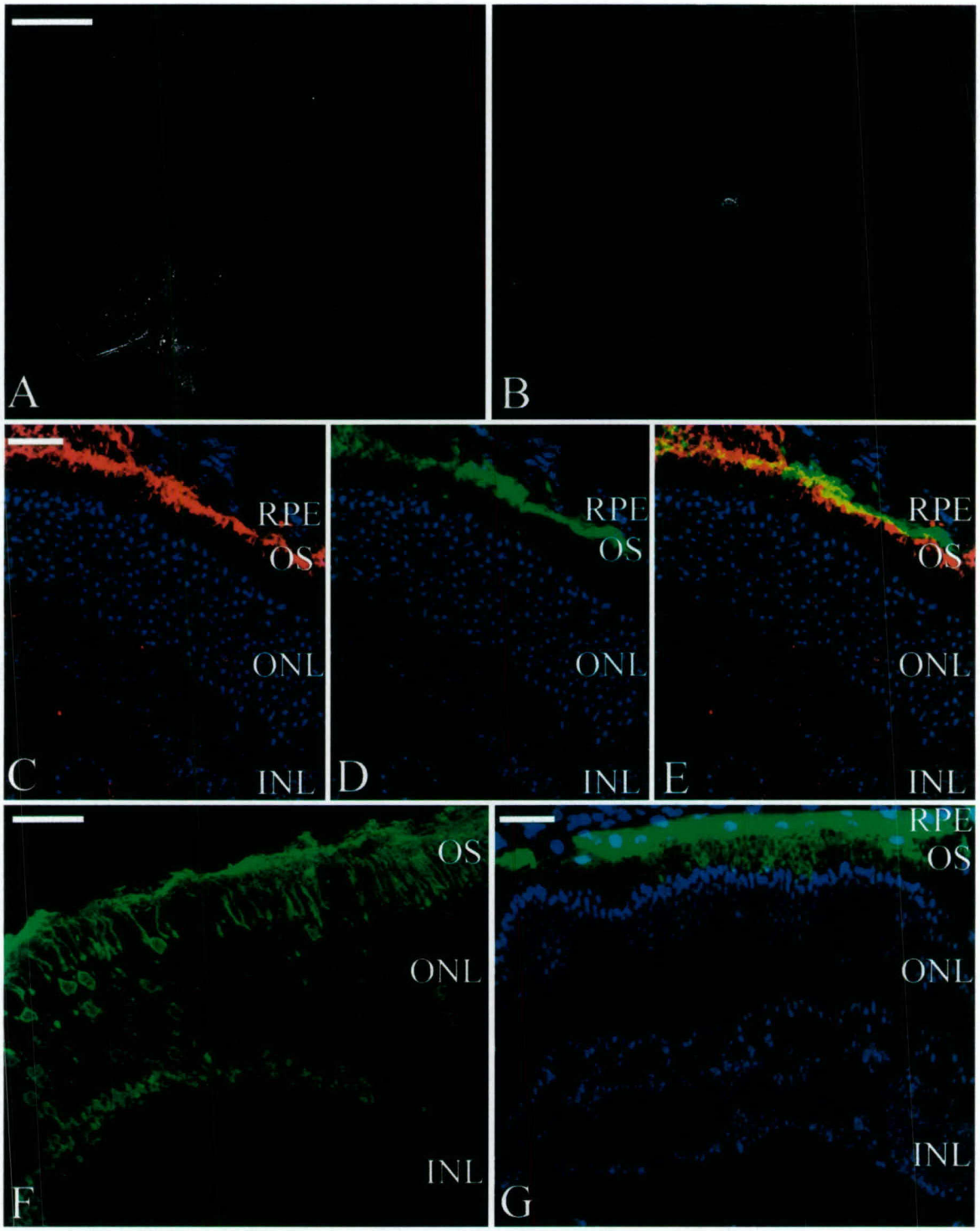

FIG. 3. GFP expression following intravitreal injection of Ad5.GFP.ΔF/37F particles. (A) Retinal whole mount from an intravitreally injected eye. Outline of the retina is indicated by the white line. (B) Retina from control (PBS-injected) eye. (C) Retinal section following intravitreal Ad5.GFP.ΔF/37F injection, stained with an anti-opsin antibody (red) and with DAPI (blue) to highlight nuclei. (D) Same section as in panel (C), showing GFP fluorescence. (E) Merge of panels (C) and (D) showing co-localization of the GFP and opsin signals. (F) Higher magnification view of an Ad5.GFP.ΔF/37F-injected retina. (G) Section of injected retina showing accumulation of GFP signal in the intercellular space and RPE. RPE, Retinal pigmented epithelium; INL, inner nuclear layer; ONL, outer nuclear layer (PR nuclei); OS, PR outer segments. Blue color, DAPI (stains all nuclei); green, GFP transgene product; red, anti-opsin (PR outer segments).

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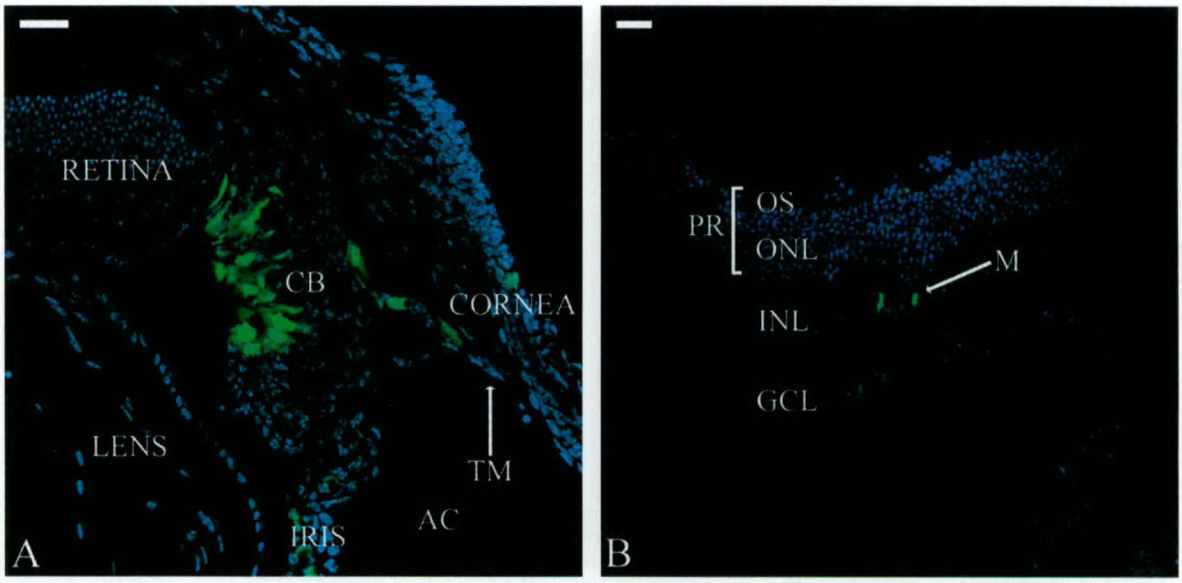

FIG. 4. EGFP-positive ciliary body, trabecular meshwork, and Müller cells after intravitreal Ad5.GFP.DF/37F injection. (A) Section of Ad5.GFP.ΔF/37F-injected eye including the ciliary body, processed as in Figure 3. (B) Retinal section of Ad5.GFP.ΔF/37F-injected eye with GFP^+ Müller cells (arrow). CB, Ciliary body; TM, trabecular meshwork; GCL, ganglion cell layer; AC, anterior chamber; PR, photoreceptors; INL, inner nuclear layer; ONL, outer nuclear layer (PR nuclei); OS, PR outer segments; M, Müller cell. Blue color, DAPI (stains all nuclei); green, GFP transgene product; red, anti-opsin (PR outer segments).

been identified as Ad37 receptors [5,36]. The PR-selective infection that we observed could be mediated by either of these, or perhaps even by a third, unrelated molecule. Interestingly, AAV vectors pseudotyped with the AAV5 capsid have been shown both to infect PR efficiently and to use sialic acid as a receptor molecule [11,40].

Previous studies suggested that the as yet unidentified Ad3 receptor is in general more widely expressed than CAR [1,3]. However, in the eye the reverse appears to be true. The pattern of infection that we observed with the Ad5-based vector Av1LacZ4 (including the iris, ciliary body, corneal endothelium, and scattered retinal neuronal cells) is in very close agreement with previous reports of intravitreal Ad5 injection in mice [19,27,37,41], rats [17], and rabbits [42]. In contrast, infection by the Ad3-pseudotyped Av9LacZ4 was largely restricted to ciliary body. Because the two viruses are identical except for their fiber genes, the differential infection most likely reflects the distribution of the relevant fiber receptors. It is also possible that the Ad3 receptor is not expressed in the eye, and that the ciliary body infection is fiber-independent (perhaps mediated exclusively by integrins).

TABLE 1: Green fluorescent protein expression in photoreceptors[a]

Virus	Number of eyes injected	Number (% of total) with $eGFP^+$ PR
Ad5.GFP.ΔF/5F	18	0 (0)
Ad5.GFP.ΔF/37F	40	11 (27)[b]

[a]Summary of photoreceptor (PR) transduction using the Ad5 or Ad37 fibers. Eyes were embedded and sectioned as described in Materials and Methods. Alternate sections were then evaluated for the presence or absence of photoreceptor EGFP signal as in Figure 3. Data presented summarize eight independent experiments with Ad5.GFP.ΔF/37F and two independent experiments with Ad5.GFP.ΔF/5F.

[b]Statistical significance was evaluated using Fisher's exact test. $P = .01$.

Intravitreal injection may be preferable to subretinal injection in the treatment of retinal degenerative disease. However, this requires that the virus traverse the entire thickness of the retina to reach PRs or RPE, which may present a barrier to access. In agreement with previous results [27], we found only very low levels of RPE transduction by an intravitreally injected Ad5 vector (Fig. 2 and data not shown). Interestingly, transgene product was much more prominent in the RPE after injection with the Ad37-pseudotyped virus (Fig. 3G), and in many sections we observed very intense fluorescence in the intercellular space between PRs and RPE. One interpretation of this observation is that the virus is traversing the entire retina and gaining access to the RPE. Another possibility is that the EGFP signal is not due to direct infection of RPE, but rather to $EGFP^+$ outer segments that have been shed from transduced PRs and phagocytosed by the RPE. GFP's tendency to be quite stable in cells [43] could allow its accumulation in RPE by this process.

Two features of the Ad37-pseudotyped particles might allow them to reach the posterior retina. First, the Ad37 fiber is much shorter (~160 versus ~350 Å) than that of Ad5 [38]. This may reduce the effective size of an Ad37-pseudotyped particle, allowing more rapid diffusion

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through the intercellular spaces. This is unlikely to explain our results completely, in that Dudus *et al.* reported that PRs were transduced after subretinal but not intravitreal injection of an AAV vector (which at 25 nm is considerably smaller than the 70-nm Ad capsid) [44]. It is also possible that because the Ad37-pseudotyped virus does not effectively use CAR [5], it might avoid interaction with cells of the anterior retina. Cells of the anterior segment (such as ganglion cells) appear to express CAR, and therefore might act as a 'sink' for CAR-binding viruses such as Ad5 vectors, preventing them from reaching the posterior retina. Another possibility is that during the injection procedure, small tears might be introduced in the retina, allowing the injected virus access to the subretinal space. However, two lines of evidence argue against this interpretation. First, we very rarely observed positive RPE cells in the >40 eyes that were injected with either Ad5.GFP.ΔF/5F or Av1LacZ4. Both of these viruses have the wild-type Ad5 fiber, and they would be expected to transduce RPE readily if they entered the subretinal space. Second, intravitreally injected Evans Blue dye was confined to the interior of the globe and was not seen in the subretinal space (data not shown).

Photoreceptor transduction is an important step in the development of therapies for retinal degenerations. Another potential application would be the delivery of antiangiogenics to the retina. PRs are well positioned to produce secreted molecules that could diffuse either anteriorly or posteriorly to target abnormal angiogenesis associated with diseases such as proliferative diabetic retinopathy or neovascular macular degeneration, respectively. Ciliary body targeting may also have clinical applications. This tissue is involved in the secretion of aqueous fluid and regulation of intraocular pressure, and represents a target for treating glaucoma. Recently, a gene product expressed in trabecular meshwork and ciliary body has been associated with certain forms of juvenile glaucoma [45]. Another application would be in rubeotic glaucoma, in which ischemic retinopathies can lead to abnormal iris neovascularization, a catastrophic complication that could potentially be treated with local expression of an anti-angiogenic gene product.

Whereas our data show that pseudotyped Ads can transduce distinct cell populations *in vivo*, we found that targeting by means of the fiber is not completely specific (for example, the Ad37-pseudotyped vector transduced RPE, ciliary body, and a few Müller cells and cells of the inner nuclear layer, along with PRs). This is an important issue, as transgene expression in an inappropriate cell type might have a deleterious effect on retinal structure or function. A combination of targeting at the level of infection and the use of a PR-specific promoter [33] should result in greater restriction of transgene expression to the desired cells. Widespread *in vivo* use of first-generation Ad vectors has been hindered by several variables, including transient gene expression and a robust immune response directed against the vector itself, but there has recently been progress toward resolving these issues. Whereas native Ad DNA exists episomally, methods have been developed to allow long-term persistence, either by integration into the target cell chromosome or by providing for episomal replication [46,47]. The 'helper-dependent' or 'gutted' Ad vectors can provide very long-term gene expression in some cases [48], and strategies to 'cloak' Ad vectors using synthetic polymers have been shown to block neutralization by antibodies [49]. Combining fiber-based targeting such as described here with advances in these other areas should lead to development of Ad-based vectors that can provide the robust gene delivery necessary for safe and efficacious clinical use.

Materials and Methods

Virus production. Av1LacZ4 and Av9LacZ4 have been described previously and were grown in 293 cells using standard techniques [3]. Both are Ad5 vectors with an RSV promoter-driven β-galactosidase reporter gene in the E1 region. Av9LacZ4 contains a chimeric fiber gene encoding a fiber that uses the Ad3 receptor rather than CAR. Ad5.GFP.ΔF is an Ad5 vector with a CMV-driven EGFP reporter gene in the E1 region and an additional deletion of the fiber gene. Stocks of this virus are propagated by growth in the 633 cell line [1], which expresses a wild-type Ad5 fiber protein. Virus for the experiments described was produced in the 633 or 714 cell lines [1,5] to generate particles containing the Ad5 or Ad37 fibers, respectively. Viruses were purified by two rounds of CsCl gradient centrifugation as described [39] and stored at −70 °C in single-use aliquots. Virus concentration was determined by assaying protein concentration (BioRad protein assay) and using the conversion factor of 1 μg protein = 4×10^9 viral particles.

Animals and injections. Animal work was carried out under protocols approved by the TSRI Department of Animal Resources. Balb/cByJ mice (6 weeks) were obtained from the TSRI breeding colony. A suspension of the indicated Ad vector or Evans Blue dye (~3 μl/eye) was injected intravitreally using a 33-gauge Hamilton needle.

Histological analysis. For GFP detection, animals were euthanized at 6 days after injection. The enucleated globes were immersed in 4% paraformaldehyde for 1 hour. Retinas were dissected and mounted, or the intact eye was washed in PBS and placed in 20% sucrose before embedding in O.C.T. for frozen sectioning. Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs) was added to label nuclei. Sections were imaged using a BioRad Confocal 1024 and images processed using Confocal Assistant (BioRad) and Photoshop (Adobe) software. For detection of β-galactosidase, eyes were harvested 7 days after infection, immersed in 2% paraformaldehyde, 0.2% glutaraldehyde for 2 hours at 4 °C, washed with PBS, and immersed overnight in staining solution (Bluo-Gal, Sigma) at 37 °C. Eyes were embedded in paraffin, and 12-μm sections were cut and counterstained with hematoxylin.

For immunostaining, whole eyes were incubated overnight in 4% paraformaldehyde at 4 °C, and frozen sections prepared as above. Sections were postfixed with Prefer (Biocare) for 30 minutes, dried for 30 minutes, and rehydrated in PBS. Sections were incubated overnight with a polyclonal anti-opsin antibody [50], washed in PBS, and treated for 1 hour with an Alexa 594-conjugated goat anti-rabbit antibody (Molecular Probes).

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